Janet D. Rowley · Michelle M. Le Beau
Terence H. Rabbitts *Editors*

Chromosomal Translocations and Genome Rearrangements in Cancer

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We hope that this volume will inform academics and clinical scientists to help their studies aimed to develop better cancer diagnostics and treatments. We are indebted to Ms. Annabel Gordon for her tireless and diligent work into the production of this volume. Without this crucial input, the book would never have been accomplished.

Contents

Part I Introduction

1 A Short History of Chromosome Rearrangements and Gene Fusions in Cancer 3
Felix Mitelman

Part II General

2 Molecular Genetics Methods in Discovery of Chromosome Structure 15
Donna G. Albertson

3 Mechanisms of Recurrent Chromosomal Translocations 27
Richard L. Frock, Jiazhi Hu, and Frederick W. Alt

4 Chromosome Translocations, Cancer Initiation and Clonal Evolution 53
Mel Greaves and Anthony M. Ford

5 Common Chromosomal Fragile Sites and Cancer 73
Yanwen Jiang, Isabelle Lucas, and Michelle M. Le Beau

6 Copy Number Changes in Carcinomas: Applications 95
Henry Wood and Pamela Rabbitts

Part III Leukaemia/Lymphoma

7 Chronic Myeloid Leukaemia 107
Debora A. Casolari and Junia V. Melo

8 Immunoglobulin and *MYC* Rearrangements inMultiple Myeloma Pathogenesis 139
P. Leif Bergsagel and W. Michael Kuehl

9 Chromosomal Translocations in B Cell Lymphomas 157
Marco Fangazio, Laura Pasqualucci, and Riccardo Dalla-Favera

10 Chromosomal Translocations and Gene Rearrangements in Acute Lymphoblastic Leukaemia..... 189
Marc R. Mansour and A. Thomas Look

11 Cellular and Molecular Basis of *KMT2A/MLL* Leukaemias: From Transformation Mechanisms to Novel Therapeutic Strategies..... 223
Bernd B. Zeisig and Chi Wai Eric So

12 Acute Promyelocytic Leukaemia: From a Specific Translocation to Cure by Targeted Therapies..... 251
Kim L. Rice and Hugues de Thé

13 Chromosome Abnormalities in Acute Myeloid Leukaemia and Their Clinical Importance 275
Krzysztof Mrózek and Clara D. Bloomfield

Part IV Sarcomas

14 Fusion Oncogenes of Sarcomas..... 321
Pierre Åman

15 Translocations in Ewing Sarcoma 333
Jason M. Tanner and Stephen L. Lessnick

Part V Epithelial Tumours

16 RET and Thyroid Carcinomas..... 357
Maria Domenica Castellone and Giancarlo Vecchio

17 Gene Fusions in Prostate Cancer..... 381
Andrew S. McDaniel and Scott A. Tomlins

18 Chromosomal Translocations in Lung Cancer..... 403
Hiroyuki Mano

19 Colon and Ovarian Translocations..... 417
Paul T. Spellman

Part VI Other Aspects

20 Pre-clinical Modelling of Chromosomal Translocations and Inversions..... 429
Katia Ruggero and Terence H. Rabbitts

21 Protein Complex Hierarchy and Translocation Gene Products 447
Jacqueline M. Matthews

22 Aberrant Transcriptional Programming in Blood Cancers 467
Constanze Bonifer, Peter N. Cockerill, and Anetta Ptasinska

Index 485

Part I
Introduction

Chapter 1
A Short History of Chromosome Rearrangements and Gene Fusions in Cancer

Felix Mitelman

Contents

1.1 Introduction 3
1.2 Chromosome Banding 4
1.3 Recombinant DNA Technology 5
1.4 Next-Generation Sequencing 7
References 9

Abstract The molecular characterization of recurrent chromosome aberrations in the early 1980s laid the foundation for gene fusion detection in cancer. This approach remained the unrivalled method to identify fusion genes for a quarter of a century and led to the detection of more than 700 neoplasia-associated fusion genes. The advancement of deep sequencing in the mid-2000s revolutionized the search for cytogenetically undetectable fusions, and such studies have dramatically changed the gene fusion landscape. A myriad of new gene fusions – more than 1,300 – the great majority involving previously unsuspected genes, have been identified by sequencing-based analyses during the past 10 years.

Keywords Cytogenetics • Karyotype • Chromosome aberrations • Gene fusions • Oncogenes

1.1 Introduction

One hundred years ago, Theodor Boveri in his famous book *Zur Frage der Entstehung maligner Tumoren* [1] proposed an idea that later became known as the somatic mutation theory of cancer, which essentially states that cancer originates in a single cell by a mitotic disturbance leading to chromosomal damage. The acquired genetic change is then propagated during subsequent mitoses to all descendants of

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the originally transformed cell. This concept is today the paradigmatic view of cancer pathogenesis, supported by a wealth of experimental evidence. It long remained a theoretical idea, however, which could not be examined critically until technical improvements in human cytogenetic analysis were made half a century later, culminating in the description of the normal human chromosome complement by Tjio and Levan in 1956 [2].

The discovery only 4 years later by Nowell and Hungerford [3] of an acquired characteristic marker chromosome consistently seen in patients with chronic myelogenous leukaemia (CML), later designated the Philadelphia chromosome (Ph) after the city where it had been found, immediately provided strong support for the idea that chromosome aberrations indeed may play a major role in the initiation of the carcinogenic process. It was reasonable to assume that the specific chromosomal abnormality – a perfect example of a somatic mutation in a haematopoietic stem cell – was the direct cause of the neoplastic state, i.e., the true verification of Boveri's somatic mutation theory. The discovery of the Ph chromosome greatly stimulated interest in cancer cytogenetics in the 1960s. However, the results obtained over the next decade were disappointing. Chromosome aberrations were detected in most tumours but no specific change comparable to the Ph was found. The abnormalities varied within the same tumour types and among patients, and at the end of the 1960s most scientists agreed that chromosome aberrations were secondary epiphenomena – not the cause, but the consequence, of neoplasia. The Ph was the exception to the rule that chromosome changes did not play any important pathogenetic role in carcinogenesis.

1.2 Chromosome Banding

The situation changed dramatically in 1970 with the introduction of chromosome banding by Caspersson and co-workers [4]. Each chromosome, chromosome arm, and even chromosome region could now be precisely identified on the basis of its unique banding pattern, and hence aberrations that previously had not been possible to detect could now be visualized. The first characteristic cytogenetic changes in cancer cells discovered with the help of the new technique appeared in 1972 (see Mitelman and Heim [5] for a review of the early data): a 14q+ marker chromosome in Burkitt lymphoma (BL), a deletion of the long arm of a chromosome 20 in polycythemia vera, +8 in acute myeloid leukaemia (AML), and −22 in meningiomas. The first balanced rearrangements were reported shortly afterwards. In 1973, Rowley first identified a reciprocal translocation between chromosomes 8 and 21, i.e., t(8;21)(q22;q22), in the bone marrow cells of a patient with AML [6] and the very same year she showed that the Ph in CML originated through a t(9;22)(q34;q11), not a deletion of the long arm of chromosome 22 as previously thought [7]. A steadily increasing number of characteristic, specific, sometimes even pathognomonic balanced rearrangements, in particular translocations, were soon described in various haematologic disorders and malignant lymphomas, including t(8;14)

(q24;q32), t(2;8)(p11;q24), and t(8;22)(q24;q11) in BL [8–11], t(15;17)(q22;q21) in acute promyelocytic leukaemia [12], t(4;11)(q21;q23) in acute lymphoblastic leukaemia [13], t(8;16)(p11;p13) in acute monocytic leukaemia [14], and t(14;18)(q32;q21) in follicular lymphoma [15]. The first specific translocations in experimental neoplasms and, as it turned out, the perfect equivalents of the characteristic rearrangements in human BL were identified by Ohno et al. [16] in mouse plasmacytomas (MPC) by the end of the 1970s.

The following decade saw a similar explosion of data emerging from studies of solid tumours, initially in particular among mesenchymal tumours. Several of the aberrations identified in the solid tumours were as specific as those previously found among haematologic malignancies, e.g., t(2;13)(q36;q14) in alveolar rhabdomyosarcoma [17], t(11;22)(q24;q12) in Ewing sarcoma [18, 19], and t(12;16)(q13;p11) in myxoid liposarcoma [20]. At this time, it also became clear that many benign tumours carried characteristic aberrations, including reciprocal translocations, e.g., t(3;8)(p21;q12) in salivary gland adenoma [21], t(3;12)(q27–28;q13–15) in lipoma [22, 23], and t(12;14)(q14;q24) in uterine leiomyoma [24–26]. All published abnormal karyotypes in neoplasia detected by banding analyses are presented in Mitelman et al. [27], and a comprehensive review of the presently known recurrent and specific chromosome aberrations may be found in Heim and Mitelman [28].

1.3 Recombinant DNA Technology

Technical developments in the late 1970s enabling the identification and characterization of genes in the breakpoints of chromosome rearrangements made it possible to elucidate the molecular consequences of the recurrent cancer-associated chromosome changes, and analyses in the early 1980s of the specific translocations in MPC, BL, and CML proved particularly pivotal for our understanding of how chromosome aberrations contribute to neoplastic transformation. When the different pieces of the puzzle were assembled, it became apparent that balanced rearrangements exert their effects by one of two mechanisms: Transcriptional up-regulation of an oncogene in one of the breakpoints through exchange of regulatory sequences in the other breakpoint, and the creation of a hybrid gene through fusion of parts of two genes, one in each breakpoint [29]. Deregulation of an oncogene by juxtaposition to a constitutively active gene region was predicted by Klein already in 1981 [30] and the principle was soon demonstrated in MPC and human BL. The breakpoints of the characteristic translocations in mice and humans were found to be located within or close to the *MYC* oncogene and one of the immunoglobulin heavy- or light-chain genes (*IGH*, *IGK* or *IGL*). As a consequence of the translocations, the entire coding part of *MYC* is juxtaposed to one of the immunoglobulin genes, resulting in deregulation of *MYC* because the gene is now driven by regulatory elements of the immunoglobulin genes. The alternative mechanism – the creation of a fusion gene – was documented at the same time in CML with the demonstration that the Ph chromosome, i.e., the der(22)t(9;22)(q34;q11), contains a fusion in which the 3′

part of the *ABL1* oncogene from 9q34 has become juxtaposed with the 5′ part of a gene from 22q11 called the *BCR* gene, resulting in the creation of an in-frame *BCR/ABL1* fusion transcript.

The first confirmation of the BL scenario in another B-cell neoplasm was the demonstration in 1984 that the t(14;18)(q32;q21) in follicular lymphoma results in overexpression of *BCL2* [31] due to its juxtaposition to the *IGH* locus, and in 1986 an analogous situation was established in T-cell acute lymphoblastic leukaemia in which regulatory elements of the T-cell receptor alpha (*TRA*) gene were found to deregulate the expression of *MYC* [32–34]; other 3′ partner genes, e.g., *LYL1*, *TAL1*, *LMO1*,and *LMO2*, involved in translocations involving *TRB* and *TRD* loci were soon identified in T-cell leukaemias/lymphomas carrying various translocations [35–39]. The CML scenario, i.e., the creation of a chimeric fusion gene, was firmly established in both haematologic malignancies and solid tumours in the early 1990s: *PML/RARA* in acute promyelocytic leukaemia with t(15;17)(q22;q21) [40, 41], *RET/CCDC6* in thyroid carcinomas with inv(10)(q11q21) [42], *DEK/NUP214* in AML with t(6;9)(p22;q34) [43], *RUNX1/RUNX1T1* in AML with t(8;21)(q22;q22) [44], and *EWSR1/FLI1* in Ewing sarcoma with t(11;22)(q24;q12) [45].

The molecular insights into the pathogenetic mechanisms of cancer-specific chromosome aberrations sparked an enormous interest in cancer cytogenetics as a powerful tool to locate and identify genes important in tumourigenesis. Further technical improvements during the 1980s, in particular the development of fluorescence in situ hybridization (FISH), multi-colour FISH, and the widespread adoption of the polymerase chain reaction (PCR), added a further sophistication to the analysis, and radically increased the precision in identifying new gene fusions [28]. This course of action – the genomic characterization of the breakpoints in cytogenetically detected specific balanced aberrations – remained the unrivalled method to identify fusion genes in cancer for a quarter of a century and led to the detection of more than 700 fusion genes (Table 1.1) caused by acquired translocations, inversions, and insertions characterizing various tumour entities [27].

Table 1.1 Gene fusions in neoplasia reported 1980–2014, based on data contained in Mitelman et al. [27]

Year	Haematologic disorders, including malignant lymphomas	Solid tumours		Total[a]
		Mesenchymal tumours	Epithelial tumours	
1980–1989	19	0	0	19
1990–1994	55	8	6	69
1995–1999	101	20	18	140
2000–2004	162	43	20	220
2005–2009	247	41	109	394
2010–2014	379	245	975	1,598
Total[a]	674	299	1,080	2,038

[a]The total numbers do not add up because each gene fusion is only counted once but may be found in distinct tumour entities

There was a major limitation of this remarkably successful approach, however. It was in principle restricted to haematological malignancies and mesenchymal tumours, which typically have simple abnormalities, often seen as a sole anomaly. Malignant epithelial tumours, representing the dominant cause of human cancer morbidity and mortality, which characteristically have complex karyotypes with numerous numerical and structural abnormalities, were consequently not amenable for analysis. As a consequence, very few fusion genes were detected in carcinomas. By 2005, only 29 fusion genes were known in carcinomas, all organs combined, as compared to 56 in mesenchymal neoplasms and 272 in haematological malignancies. These quantitative differences led to the generally held view that fusion genes are not an important mechanism in carcinoma pathogenesis. Indirect evidence that fusion genes actually may play the same fundamental role in epithelial carcinogenesis as they do for the initiation of haematologic and mesenchymal neoplasms was presented by Mitelman et al. [46], and direct evidence clearly substantiating this view was soon produced with the help of new powerful technologies developed during the last decade.

1.4 Next-Generation Sequencing

The breakthrough in the search for fusion genes by alternative methods to chromosome banding analysis followed by reverse transcriptase-PCR and Sanger sequencing was made by Chinnaiyan and coworkers in 2005 [47]. They took a bioinformatics approach to look for genes in prostate cancer that showed a very high expression in RNA microarray experiments, and demonstrated that two of the outlier genes – *ERG* and *ETV1* – were frequently fused to the 5′ part of the prostate-specific androgen-regulated gene *TMPRSS2*. Subsequently other *ETS* family genes were found to be fused with *TMPRSS2*, and several other 5′ partner genes that activate *ETS* genes were also discovered [48]. The frequencies of the various fusions vary slightly in different patient series depending on the populations studied but altogether about 80 % of prostate cancers harbour one of the presently known fusion genes, the most common being *TMPRSS2*/*ERG*. Very soon afterwards, an *EML4*/*ALK* gene fusion was found in a subset of non-small cell lung cancer by screening a retroviral cDNA expression library from cancer samples [49]. The importance of these results in prostate and lung cancer cannot be overestimated. They showed, for the first time, that cytogenetically undetectable gene fusions may be a causative factor in a substantial fraction of common human cancers, and the findings underscored the need for high-resolution methods to be used in parallel with chromosome banding to characterize cancer genomes. The advancement of next-generation sequencing (NGS) at this time revolutionized the search for new fusion genes, enabling unprecedented opportunities to process thousands of tumours for systematic mutation and fusion gene discovery without any knowledge of the genetic constitution. The first report using the new sequencing technology to find fusion genes in cancer was presented by Stratton and co-workers in 2008 [50].

Numerous studies of common cancer types, such as carcinomas of the breast, lung, prostate, and uterus, quickly followed (e.g., [51–57]), and the results have dramatically changed the gene fusion landscape. A myriad of new gene fusions – more than 1,300 – the great majority involving previously unsuspected genes, have been identified with the help of NGS-based analysis [27]. Table 1.1 shows the dramatic increase of gene fusions detected since 2010, in particular among malignant epithelial tumours, and Table 1.2 presents the distribution of all presently reported fusions among major neoplasia subtypes.

As can be seen from Table 1.1, the total number of gene fusions now exceeds 2,000 and at least 65 % of these were identified by various sequencing technologies during the last 5 years. Clearly, the presently known gene fusions represent only the tip of an iceberg. Given the extraordinary rate at which The Cancer Genome Atlas (TCGA) project is generating cancer genomic data [58, 59], a huge number of new

Table 1.2 Number of gene fusions and genes involved in fusions in major neoplasia subtypes, based on Mitelman et al. [27]

Diagnosis	Number of gene fusions	Number of genes involved in fusions
Haematologic disorders		
Undifferentiated and biphenotypic leukaemia	24	32
Acute myeloid leukaemia	267	339
Myelodysplastic syndromes	50	59
Chronic myeloproliferative disorders, including CML	68	84
Acute lymphoblastic leukaemia	192	188
Plasma cell neoplasms	20	23
Mature B-cell neoplasms	179	195
Mature T- and NK-cell neoplasms	28	37
Hodgkin lymphoma	13	19
Solid tumours		
Benign solid tumours		
Benign epithelial tumours	14	20
Benign mesenchymal tumours	37	58
Malignant solid tumours		
Respiratory system	373	596
Digestive system	62	109
Breast	343	578
Female genital organs	95	185
Male genital organs	142	209
Urinary tract	55	98
Endocrine system	22	28
Nervous system	131	227
Skin	12	24
Bone	24	30
Soft tissues	81	105

genomic rearrangements can be expected to be discovered within the next few years. It is important in this context to mention two notable differences between the fusion genes detected on the basis of cytogenetically identified aberrations and those so far identified by NGS. First, multiple NGS-detected fusion genes are generally found within the same tumour, e.g., more than 25 different fusions in one prostate cancer, and secondly, very few of the NGS-detected fusion genes have been found to be recurrent. A major challenge will be to verify by functional studies which of the alleged gene fusions are primary, pathogenetically important, and which are either secondary progressional changes or non-consequential "noise" abnormalities.

References

1. Boveri T (1914) Zur Frage der Entstehung maligner Tumoren. Gustav Fisher Verlag, Jena
2. Tjio JH, Levan A (1956) The chromosome number of man. Hereditas 42:1–6
3. Nowell PC, Hungerford DA (1960) A minute chromosome in human chronic granulocytic leukemia. Science 132:1497
4. Caspersson T, Zech L, Johansson C (1970) Differential binding of alkylating fluorochromes in human chromosomes. Exp Cell Res 60:315–319
5. Mitelman F, Heim S (2015) How it all began: cancer cytogenetics before sequencing. In: Heim S, Mitelman F (eds) Cancer cytogenetics, 4th edn. Wiley, New York
6. Rowley JD (1973) Identification of a translocation with quinacrine fluorescence in a patient with acute leukemia. Ann Genet 16:109–112
7. Rowley JD (1973) A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. Nature 243:290–293
8. Zech L, Haglund U, Nilsson K, Klein G (1976) Characteristic chromosomal abnormalities in biopsies and lymphoid-cell lines from patients with Burkitt and non-Burkitt lymphomas. Int J Cancer 17:47–56
9. Berger R, Bernheim A, Weh H-J, Flandrin G, Daniel MT et al (1979) A new translocation in Burkitt's tumor cells. Hum Genet 53:111–112
10. Miyoshi I, Hiraki S, Kimura I, Miyamoto K, Sato J (1979) 2/8 translocation in a Japanese Burkitt's lymphoma. Experientia 35:742–743
11. van den Berghe H, Parloir C, Gosseye S, Englebienne V, Cornu G et al (1979) Variant translocation in Burkitt lymphoma. Cancer Genet Cytogenet 1:9–14
12. Rowley JD, Golomb HM, Dougherty C (1977) 15/17 translocation, a consistent chromosomal change in acute promyelocytic leukaemia. Lancet 1:549–550
13. Oshimura M, Freeman AI, Sandberg AA (1977) Chromosomes and causation of human cancer and leukemia. XXVI. Banding studies in acute lymphoblastic leukemia (ALL). Cancer 40:1161–1172
14. Mitelman F, Brandt L, Nilsson PG (1978) Relation among occupational exposure to potential mutagenic/carcinogenic agents, clinical findings, and bone marrow chromosomes in acute nonlymphocytic leukemia. Blood 52:1229–1237
15. Fukuhara S, Rowley JD, Variakojis D, Golomb HM (1979) Chromosome abnormalities in poorly differentiated lymphocytic lymphoma. Cancer Res 39:3119–3128
16. Ohno S, Babonits M, Wiener F, Spira J, Klein G et al (1979) Nonrandom chromosome changes involving the Ig gene-carrying chromosomes 12 and 6 in pristane-induced mouse plasmacytomas. Cell 18:1001–1007
17. Seidal T, Mark J, Hagmar B, Angervall L (1982) Alveolar rhabdomyosarcoma: a cytogenetic and correlated cytological and histological study. APMIS 90:345–354

18. Aurias A, Rimbaut C, Buffe D, Dubousset J, Mazabraud A (1983) Chromosomal translocations in Ewing's sarcoma. N Engl J Med 309:496–497
19. Turc-Carel C, Philip I, Berger M-P, Philip T, Lenoir GM (1983) Chromosomal translocations in Ewing's sarcoma. N Engl J Med 309:497–498
20. Limon J, Turc-Carel C, Dal Cin P, Rao U, Sandberg AA (1986) Recurrent chromosome translocations in liposarcoma. Cancer Genet Cytogenet 22:93–94
21. Mark J, Dahlenfors R, Ekedahl C, Stenman G (1980) The mixed salivary gland tumor – a normally benign human neoplasm frequently showing specific chromosomal abnormalities. Cancer Genet Cytogenet 2:231–241
22. Heim S, Mandahl N, Kristoffersson U, Mitelman F, Rööser B et al (1986) Reciprocal translocation t(3;12)(q27;q13) in lipoma. Cancer Genet Cytogenet 23:301–304
23. Turc-Carel C, Dal Cin P, Rao U, Karakousis C, Sandberg AA (1986) Cytogenetic studies of adipose tissue tumors. I. A benign lipoma with reciprocal translocation t(3;12)(q28;q14). Cancer Genet Cytogenet 23:283–289
24. Heim S, Nilbert M, Vanni R, Floderus U-M, Mandahl N et al (1988) A specific translocation, t(12;14)(q14-15;q23-24), characterizes a subgroup of uterine leiomyomas. Cancer Genet Cytogenet 32:13–17
25. Mark J, Havel G, Grepp C, Dahlenfors R, Wedell B (1988) Cytogenetical observations in human benign uterine leiomyomas. Anticancer Res 8:621–626
26. Turc-Carel C, Dal Cin P, Boghosian L, Terk-Zakarian J, Sandberg AA (1988) Consistent breakpoints in region 14q22-q24 in uterine leiomyoma. Cancer Genet Cytogenet 32:25–31
27. Mitelman F, Johansson B, Mertens F (2014) Mitelman database of chromosome aberrations and gene fusions in cancer. http://cgap.nci.nih.gov/Chromosomes/Mitelman
28. Heim S, Mitelman F (eds) (2015) Cancer cytogenetics, 4th edn. Wiley, New York
29. Mitelman F, Johansson B, Mertens F (2007) The impact of translocations and gene fusions on cancer causation. Nat Rev Cancer 7:233–245
30. Klein G (1981) The role of gene dosage and genetic transpositions in carcinogenesis. Nature 294:313–318
31. Tsujimoto Y, Finger LR, Yunis J, Nowell PC, Croce CM (1984) Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. Science 226:1097–1099
32. Erikson J, Finger L, Sun L, ar-Rushdi A, Nishikura K et al (1986) Deregulation of c-myc by translocation of the alfa-locus of the T-cell receptor in T-cell leukemias. Science 232:884–886
33. Mathieu-Mahul D, Sigaux F, Zhu C, Bernheim A, Mauchauffe M et al (1986) A t(8;14)(q24;q11) translocation in a T-cell leukemia (L1-ALL) with c-myc and TcR-alpha chain locus rearrangements. Int J Cancer 38:835–840
34. McKeithan TW, Shima EA, Le Beau MM, Minowada J, Rowley JD et al (1986) Molecular cloning of the breakpoint junction of a human chromosomal 8;14 translocation involving the T-cell receptor alpha-chain gene and sequences on the 3′ side of MYC. Proc Natl Acad Sci U S A 83:6636–6640
35. Boehm T, Baer R, Lavenir I, Forster A, Waters JJ et al (1988) The mechanism of chromosomal translocation t(11;14) involving the T-cell receptor Cdelta locus on human chromosome 14q11 and a transcribed region of chromosome 11p15. EMBO J 7:385–394
36. Mellentin JD, Smith SD, Cleary ML (1989) lyl-1, a novel gene altered by chromosomal translocation in T cell leukemia, codes for a protein with a helix-loop-helix DNA binding motif. Cell 58:77–83
37. Finger LR, Kagan J, Christopher G, Kurtzberg J, Hershfield MS et al (1989) Involvement of the TCL5 gene on human chromosome 1 in T-cell leukemia and melanoma. Proc Natl Acad Sci U S A 86:5039–5043
38. McGuire EA, Hockett RD, Pollock KM, Bartholdi MF, O'Brien SJ et al (1989) The t(11;14)(p15;q11) in a T-cell acute lymphoblastic leukemia cell line activates multiple transcripts, including Ttg-1, a gene encoding a potential zinc finger protein. Mol Cell Biol 9:2124–2132

39. Boehm T, Foroni L, Kaneko Y, Perutz MF, Rabbitts TH (1991) The rhombotin family of cysteine-rich LIM-domain oncogenes: distinct members are involved in T-cell translocations to human chromosomes 11p15 and 11p13. Proc Natl Acad Sci U S A 88:4367–4371
40. Lemons RS, Eilender D, Waldmann RA, Rebentisch M, Frej A-K et al (1990) Cloning and characterization of the t(15;17) translocation breakpoint region in acute promyelocytic leukemia. Gene Chromosome Cancer 2:79–87
41. de Thé H, Chomienne C, Lanotte M, Degos L, Dejean A (1990) The t(15;17) translocation of acute promyelocytic leukaemia fuses the retinoic acid receptor alpha gene to a novel transcribed locus. Nature 347:558–561
42. Pierotti MA, Santoro M, Jenkins RB, Sozzi G, Bongarzone I et al (1992) Characterization of an inversion on the long arm of chromosome 10 juxtaposing D10S170 and RET and creating the oncogenic sequence RET/PTC. Proc Natl Acad Sci U S A 89:1616–1620
43. von Lindern M, Fornerod M, van Baal S, Jaegle M, de Wit T et al (1992) The translocation (6;9), associated with a specific subtype of acute myeloid leukemia, results in the fusion of two genes, dek and can, and the expression of a chimeric, leukemia-specific dek-can mRNA. Mol Cell Biol 12:1687–1697
44. Erickson P, Gao J, Chang K-S, Look T, Whisenant E et al (1992) Identification of breakpoints in t(8;21) acute myelogenous leukemia and isolation of a fusion transcript, AML1/ETO, with similarity to Drosophila segmentation gene, runt. Blood 80:1825–1831
45. Delattre O, Zucman J, Plougastel B, Desmaze C, Melot T et al (1992) Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours. Nature 359:162–165
46. Mitelman F, Johansson B, Mertens F (2004) Fusion genes and rearranged genes as a linear function of chromosome aberrations in cancer. Nat Genet 36:331–334
47. Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R et al (2005) Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science 310:644–648
48. Rubin MA, Maher CA, Chinnaiyan AM (2011) Common gene rearrangements in prostate cancer. J Clin Oncol 29:3659–3668
49. Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y et al (2007) Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature 448:561–566
50. Campbell PJ, Stephens PJ, Pleasance ED, O'Meara S, Li H et al (2008) Identification of somatically acquired rearrangements in cancer using genome-wide massively parallel paired-end sequencing. Nat Genet 40:722–729
51. Maher CA, Kumar-Sinha C, Cao X, Kalyana-Sundaram S, Han B et al (2009) Transcriptome sequencing to detect gene fusions in cancer. Nature 458:97–101
52. Stephens PJ, McBride DJ, Lin M-L, Varela I, Pleasance ED et al (2009) Complex landscapes of somatic rearrangement in human breast cancer genomes. Nature 462:1005–1010
53. Robinson DR, Kalyana-Sundaram S, Wu Y-M, Shankar S, Cao X et al (2011) Functionally recurrent rearrangements of the MAST kinase and Notch gene families in breast cancer. Nat Med 17:1646–1651
54. Asmann YW, Necela BM, Kalari KR, Hossain A, Baker TR et al (2012) Detection of redundant fusion transcripts as biomarkers or disease-specific therapeutic targets in breast cancer. Cancer Res 72:1921–1928
55. Hammerman PS, Hayes DN, Wilkerson MD, Schultz N, Bose R et al (2012) Comprehensive genomic characterization of squamous cell lung cancers. Nature 489:519–525
56. Lapuk AV, Wu C, Wyatt AW, McPherson A, McConeghy BJ et al (2012) From sequence to molecular pathology, and a mechanism driving the neuroendocrine phenotype in prostate cancer. J Pathol 227:286–297
57. Kandoth C, Schultz N, Cherniack AD, Akbani R, Liu Y et al (2013) Integrated genomic characterization of endometrial carcinoma. Nature 497:67–73
58. Kandoth C, McLellan MD, Vandin F, Ye K, Niu B et al (2013) Mutational landscape and significance across 12 major cancer types. Nature 402:333–341
59. Weinstein JN, Collisson EA, Mills GB, Mills Shaw KR, Ozenberger BA et al (2013) The cancer genome atlas pan-cancer analysis project. Nat Genet 45:1113–1120

Part II
General

Chapter 2
Molecular Genetics Methods in Discovery of Chromosome Structure

Donna G. Albertson

Contents

2.1 Introduction 16
2.1.1 Cancer Genome Alterations 16
2.1.2 Cytogenetic and Molecular Techniques 18
2.1.2.1 Fluorescent *In Situ* Hybridization (FISH) 18
2.1.2.2 Comparative Genomic Hybridization 19
2.1.2.3 Amplification-Based Methods for Genome Copy Number Measurement 20
2.1.2.4 Multiplex Ligation-Dependent Probe Amplification (MLPA) 20
2.1.2.5 nCounter® 21
2.1.2.6 Whole Genome Sequencing 21
2.1.3 Combining Technologies to Better Study Tumours 22
References 24

Abstract Somatically acquired tumour genome alterations underlie many of the changes in gene expression that promote tumour formation. These changes, ranging from single nucleotide changes to those involving parts of chromosomes or whole chromosomes, likely reflect the many different solutions taken by individual tumours to escape normal growth regulatory mechanisms. A variety of molecular and cytogenetic techniques, differing in resolution and capabilities for high throughput or single cell analysis, for example, have been used to investigate the altered state of tumour genomes. Some of these methods have become the mainstay of clinical cancer diagnosis and patient management.

Keywords Copy number • Chromosome aberrations • FISH • CGH • Whole genome sequencing

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2.1 Introduction

Human cancer genomes typically have somatically acquired genome alterations, spanning the range from single nucleotide changes to those involving parts of chromosomes or whole chromosomes. These aberrations underlie many of the changes in gene expression that promote tumour formation, such as increases or decreases in copy number of coding regions, inactivation or activation of genes by point mutations or gene disruption, and activation of genes by mutation and rearrangements that create fusion genes with new properties and gene expression patterns. A variety of molecular and cytogenetic techniques have been used to investigate the altered state of tumour genomes, some of which have become the mainstay of clinical cancer diagnosis and patient management.

2.1.1 Cancer Genome Alterations

Great variation in the numbers and types of chromosome level alterations present in human tumours has been reported, which is likely to reflect the many different solutions taken by individual tumours to escape normal growth regulatory mechanisms. Figure 2.1 illustrates chromosome level alterations that may occur in a tumour genome.

Some changes result in loss of heterozygosity (LOH), i.e., change in the normal equal contribution to the diploid genome from both the maternal and paternal chromosomes. Such alterations do not affect the number of copies of regions of the genome and are often referred to as "copy neutral" changes; however, gene expression may be altered by LOH and contribute to tumour formation. For example, at a locus heterozygous for a mutation in a tumour suppressor gene, somatic recombination could result in loss of the wild type allele and its replacement with the mutant copy, such that the cell would be homozygous for the mutation. Intra-chromosomal inversions, another class of copy neutral alterations might also alter gene expression as a result of a change in the gene's neighbourhood or fusion with part of another gene, for example.

By contrast, chromosome level alterations often result in net gain or loss of whole chromosomes (aneuploidy) or parts of chromosomes (insertions, deletions, non-reciprocal translocations). Gene amplification, defined as a copy number increase of a restricted region of a chromosome arm, may also occur [1]. The analysis of amplified DNA in mammalian cell lines and tumours has revealed that it may be organized as extra chromosomal copies, called double minutes, in tandem arrays as head to tail or inverted repeats within a chromosome, often forming a cytologically visible homogeneously staining region (HSR) or distributed at multiple locations in the genome [1]. The unit of amplified DNA in some cases may involve sequences from two or more regions of the genome, indicating a more complex process of formation involving multiple chromosomes [2]. Regions of focal copy

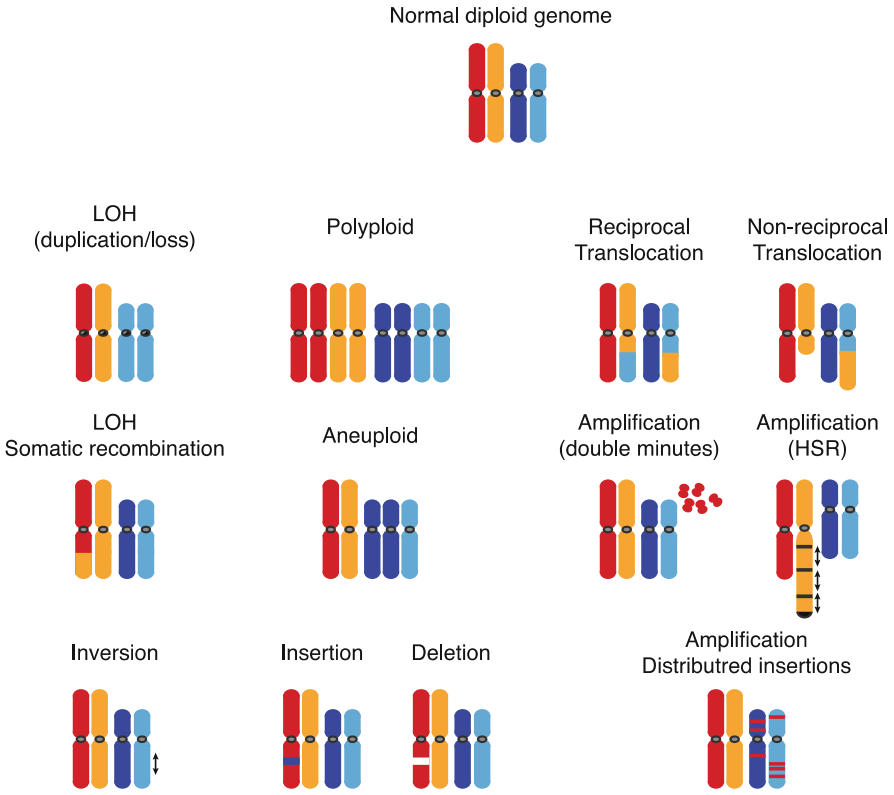

Fig. 2.1 Schematic illustration of mechanisms by which chromosomal aberrations arise. A two chromosome diploid genome with a large (*red* and *orange*) and smaller chromosome (*blue* and *light blue*) pair is depicted at the *top* of the figure. The maternal and paternal chromosomes of the pairs are distinguished by shades of *red*/*orange* and *blue*/*light blue*

number alterations, such as small deletions and amplicons focus attention on genes in these regions as potential tumour suppressors and oncogenes, respectively. Regions of amplification pinpoint genes whose elevated expression is likely to be beneficial to the tumour. As amplicons are unstable [3], it is likely that there is ongoing selection for their retention and the elevated expression of gene(s) in the region.

Cytogenetic and molecular methods have been applied to study the organization of frequently amplified oncogenes such as *MYCN*, *EGFR* and *ERBB2* [4–6]. Recently, application of allele specific copy number and high throughput sequencing technologies (see below) has provided fine resolution maps of amplicons and chromosome alterations in tumours. The term "chromothripsis" (derived from Greek, chromo for chromosome and thripsis, shattering to pieces) was used to describe the complex rearrangements involving multiple breakpoints and copy number alterations seen in 2–3 % of cancers [7]. It was imagined that the rearrangements occurred in a single cataclysmic event, which involved shattering of a

chromosome and its reassembly, rather than stepwise accumulation over time, the existing view of genome evolution in cancer. Although the term gained popularity and has even been considered a mechanism, its usage was subsequently appropriately criticized based on mathematical modelling and existing knowledge of cancer genomes and nuclear organization [8, 9]. Indeed, the complex amplicons that might be formed via a breakage-fusion-bridge process [10], in which repeated cycles of fusion of broken ends of chromosomes lead to failure to segregate properly at mitosis with subsequent breakage during anaphase, were considered examples of chromothripsis. Such a process, however, requires multiple rounds of cell division, generates unstable chromosomes [3], and results in heterogeneity in a population of cells, consistent with the observed copy number profiles characteristic of chromothripsis [9]. In summary, therefore, there appears to be little need for this terminology, the observed complex rearrangements being adequately explained by known genomic instability mechanisms.

2.1.2 Cytogenetic and Molecular Techniques

2.1.2.1 Fluorescent *In Situ* Hybridization (FISH)

A variety of cytogenetic applications use FISH to detect changes in the copy number of loci, a change in the organization of the loci on a chromosome (e.g., inversions, deletions duplications, amplifications) and between chromosomes (e.g., translocations, amplifications). The method uses one or more nucleic acid probes labelled with a fluorochrome conjugated nucleotide or other hapten, such as biotin, that can be detected by fluorescently labelled molecules such as avidin or hapten specific antibodies. The labelled probe(s) are hybridized to whole organisms, tissue sections, cells or subcellular constituents such as metaphase chromosomes, nuclei or extended chromatin fibres and the site of the nucleic acid sequence visualized by fluorescence microscopy [11, 12]. Single locus FISH probes are currently in routine use in clinical laboratories, for example, to assess amplification of *ERBB2* in tumours as a guide to therapeutic decisions [13] and to detect aneuploid cells in urine as a non-invasive alternative to cystoscopy to monitor bladder cancer patients for disease recurrence [14]. Recurrent translocations characteristic of certain cancers are also used to identify cancers. Probes that flank the breakpoints are labelled in different colours and following hybridization, the presence of the translocation is readily observed by separation of the two normally overlapping coloured signals in the cancer cell nuclei [15, 16].

Analysis of tumour karyotypes using FISH to differentially label whole chromosomes or parts of chromosomes (painting probes) can provide higher resolution information on chromosome rearrangements than is possible by standard G-bands by Trypsin using Giemsa (GTG) metaphase chromosome analysis, especially for the common situation in which it is not possible to prepare well banded metaphases from the tumour. Two of the first described painting probe approaches, Spectral

karyotyping (SKY) and M-FISH developed for this purpose used chromosome specific probe libraries differentially labelled with four to seven different fluorophores [17, 18]. Following hybridization and imaging, sequences from the 24 human chromosomes can be distinguished based on the spectroscopic properties of the probes and localized on metaphase spreads prepared from the tumour. Aneuploidies and the composition of abnormal marker chromosomes can be revealed using whole chromosome paints, but within chromosome structural aberrations, such as inversions, deletions, insertions, and duplications cannot be detected. Variations on labelling with whole chromosome paints include, for example, addition of region specific probes obtained by chromosome microdissection or locus specific probes [19]. These alternatives can provide higher resolution information on specific genome regions or types of aberrations.

2.1.2.2 Comparative Genomic Hybridization

Described in 1992, comparative genomic hybridization (CGH) provided the first efficient approach to scan the entire genome for variations in DNA copy number [20]. In the original implementation of CGH, total genomic DNAs isolated from test and reference cell populations were differentially labelled and hybridized to metaphase chromosomes. The binding of sequences at different genomic locations was measured relative to the physical position on the chromosomes. Subsequently, as the human genome mapping and sequencing projects progressed, chromosomes were replaced by DNA microarrays containing elements, initially bacterial artificial chromosome (BAC) [21] or cDNA [22] clones spanning the genome, which had been mapped directly to the physical map of the genome or genome sequence. With either representation of the genome, copy number is determined from the relative hybridization intensity of the test and reference signals at a given genomic location and is proportional to the relative copy number of those sequences in the test and reference genomes. Typically, the reference sample has a normal genome, so that increases and decreases in ratio directly indicate DNA copy number variation in the genome of the test sample. Data are typically normalized so that the modal ratio for the genome is set to some standard value, typically 1.0 on a linear scale or 0.0 on a logarithmic scale. With the completion of the human genome sequence, arrays comprised of short oligonucleotides containing single nucleotide polymorphisms (SNP arrays) became commercially available that allowed information on allele specific copy number to be obtained [23, 24], thereby providing data on both copy number and LOH. At present, whole genome sequencing is replacing microarray-based methods for measuring copy number (see below).

An alternative comparative genomic hybridization platform uses molecular inversion probes (MIPs). Available as the Oncoscan™ FFPE Assay from Affymetrix, Inc., the technology can be used to detect selected cancer relevant single nucleotide mutations and measures copy number and LOH with 300 kb resolution from small amounts of DNA extracted from frozen or FFPE material [25]. The technology uses padlock probes [26]. The probes are designed such that the two ends of the probes

hybridize to ~40 bp regions in the genome leaving a single nucleotide gap. The gap is filled (allowing SNP detection) and the ends of the probes ligated to generate circular probes. Exonuclease digestion is used to remove other nucleic acid sequences and the probes are hybridized to an array via a specific tag sequence included in each probe. Copy number is then determined relative to a normal reference, ideally a patient matched normal sample. Advantages of the technology include requirement for small amounts of sample DNA, compatibility with degraded DNA extracted from FFPE samples, and simultaneous copy number, LOH and SNP detection. A version of the technology is the first chromosomal microarray to receive FDA approval for postnatal testing for germline chromosomal copy number alterations associated with developmental delay, intellectual disability, congenital anomalies, and/or dysmorphic features.

2.1.2.3 Amplification-Based Methods for Genome Copy Number Measurement

Real time quantitative polymerase chain reaction (PCR) has been used to measure copy number at specific loci in the genome relative to a reference locus. An advantage of this approach is the rapid turnaround time and possibility of automating the assay for hundreds of samples. The choice of reference locus for studies of cancer genomes, however, can be problematic. The copy number of the reference locus may not be known, it may not be single copy in tumours and the copy number may vary amongst tumours in a cohort under study. To address this problem, a multicopy reference has been introduced (Qiagen, Inc.). A reference sequence present in >20 copies per diploid genome and distributed across the genome is relatively insensitive to changes in copy number that affect a single locus or a few of the loci, and gain or loss of one or a few copies will result in only a small change in the measured C_T value for the reference.

2.1.2.4 Multiplex Ligation-Dependent Probe Amplification (MLPA)

The MLPA method measures copy number in a multiplex polymerase chain reaction [27]. Locus specific probes recognizing adjacent regions in the genome are annealed, ligated and amplified using universal primer sequences. Probe sets are designed such that ~50 amplification products can be distinguished and quantified following separation by capillary electrophoresis. Comparison of a test sample to a reference sample provides information on copy number. Probes can also be designed to interrogate SNPs and methylation status [28]. While MLPA offers advantages in terms of cost, turnaround time and capability to use degraded FFPE DNA, it is limited to the simultaneous analysis of ~50 loci. Performance of MLPA is also sensitive to the choice of reference DNA, ideally it should be normal DNA from the same individual extracted in the same manner as the test sample [28].

2.1.2.5 nCounter®

The nCounter® system from NanoString® captures and directly counts individual molecules without the need for amplification and can be used to determine copy number at defined loci in the genome [29]. The system uses a 35–50 base pair capture probe complementary to a nucleic acid sequence of interest and a second 35–50 base pair reporter probe complementary to a second region of the nucleic acid sequence of interest. The reporter probe carries a coloured barcode consisting of a DNA sequence annealed with complementary in vitro transcribed RNA sequences each labelled with a fluorophore. Multiplex hybridization of the region specific probes takes place in solution. Following hybridization, excess probe is washed away and the hybridized complexes are oriented and extended on a capture surface by application of an electric field. The linear order of the fluorophores in the barcodes of single molecules are then imaged and counted to determine the copy number of each locus. The nCounter® system allows simultaneous interrogation of several hundred loci and is suitable for use with DNA obtained from fresh frozen or FFPE samples.

2.1.2.6 Whole Genome Sequencing

Next generation sequencing (NGS) or high throughput whole genome sequencing technology offers the opportunity to sequence millions of reads in a cost effective manner. Four general methods are used to identify copy number alterations using NGS, including assembly-based methods, depth of coverage or read depth methods, paired-end or read-pair and split-read methods [30, 31]. Assembly-based methods, which reconstruct a genome de novo are best suited to studies of small genomes and have not been widely applied in human genome studies. The other three methods rely on aligning sequence reads to a previously established reference genome for the organism.

Depth of coverage methods (DOC) use short single or paired end reads and determine copy number based on number of reads that fall within a bin of defined size, e.g., 15 kb. There is an underlying assumption of uniform sequence coverage of the genome; however, the variation of counts amongst bins is affected by the DNA copy number variations, the Poisson statistics of counting reads, and by biases of the analytical process that have substantial dependences on such factors as the GC content and mappability of sequences in the bins. Coverage is reduced in regions of the genome with high or low GC content and in repetitive regions in which reads cannot be mapped unambiguously. Algorithms to correct for these biases have been developed. Alternatively, comparisons to sequencing data from appropriate reference genomes have been used to normalize data from test samples. Algorithms incorporating information on SNP heterozygosity have also been used to call both copy number and loss of heterozygosity. The capability of DOC methods to use short single end reads offers an advantage when working with archival FFPE tumour specimens from which DNA is likely to be fragmented. A further benefit for tumour

genome analysis is the use of reference free DOC methods, since matched normal reference DNA may not be available [32].

Paired end and split read methods require paired sequencing reads. Deletions and insertions are detected when the paired reads align to the reference genome at distances greater than or less than expected, respectively based on the length of the fragments being sequenced. Paired-end sequencing can also detect inversions and translocations depending on the manner in which the paired ends map to the reference genome. Breakpoints in the genome can be quite accurately mapped by analysis of split-reads in the case that one of the paired end reads maps to the sequence and the other read, which fails to align is considered to span the breakpoint of a genome rearrangement.

A number of algorithms have been described for detecting copy number alterations from NGS data. They address the general workflow of first inferring copy number profiles from the raw sequence, segmenting the profiles and calling aberrations. A comparison of algorithms revealed differences in sensitivity and specificity for different sizes and types of genome alterations [33]. Further refinements in algorithms for detecting tumour genome copy number and structure are expected to better address the technical biases inherent in the current sequencing methodology, as well as incorporating improved knowledge of human genome variation to identify germline copy number variants that could be misinterpreted as tumour genome alterations [34].

2.1.3 Combining Technologies to Better Study Tumours

Cytogenetic and molecular methods for detecting and measuring tumour genome alterations vary in resolution, utility for detecting previously unknown aberrations and sensitivity to admixed normal cells or tumour heterogeneity. The combined use of more than one technique can provide greater insight into alterations in the genomes of the tumour cells. An example is shown in Fig. 2.2, in which array CGH and FISH were used to study an oral squamous cell carcinoma (SCC) primary and recurrence. Analysis of the primary and recurrence by array CGH revealed low level gains and losses in the genomes, as well as amplification of *CCND1* on chromosome 11. By contrast, amplification of *EGFR* on chromosome 7 was observed only in the primary (Fig. 2.2a). Using FISH probes for *EGFR* and *CCND1*, amplification of both regions was apparent in the primary, but *EGFR* was only modestly elevated in the recurrence (Fig. 2.2b), consistent with the array CGH copy number analysis. Enumeration of FISH counts at five different regions in the primary, however, revealed that the tumour was heterogeneous with respect to amplification of *EGFR* with one of five regions having only modestly elevated copy number of *EGFR* similar to the recurrence (Fig. 2.2c). These observations suggest that the recurrence possibly originated from (residual) cells from this region lacking *EGFR* amplification.

In the above example, specific chromosome alterations were selected for analysis based on the genome-wide copy number information provided by array CGH. By

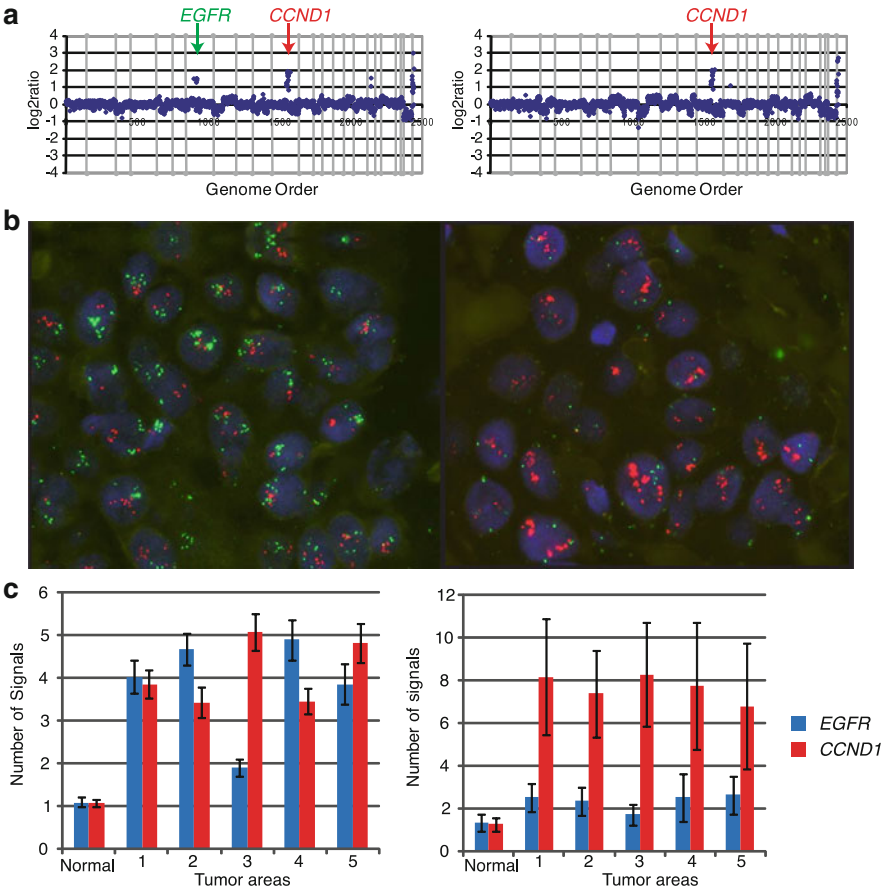

Fig. 2.2 Combining FISH and array CGH reveals tumour heterogeneity. (**a**) Copy number profiles of an oral SCC primary (*left*) and recurrence (*right*). The normalized $\log_2$ ratio is plotted at each locus sorted by chromosome and ordered according to genome position from the p-arm to the q-arm. Amplifications of *EGFR* on chromosome 7 and *CCND1* on chromosome 11 are present in the primary, but only amplification of *CCND1* in the recurrence. (**b**) Hybridization of FISH probes for *EGFR* (*green*) and *CCND1* (*red*) to tissue sections from the primary (*left*) and recurrence (*right*). The large clusters of green signals indicative of *EGFR* amplification are absent from the recurrence consistent with the array CGH profiles. (**c**) Enumeration of FISH signals from five regions of the sections from the primary (*left*) and (recurrence) *right*. While four of the five analyzed regions showed elevated counts for both *EGFR* and *CCND1* in the primary (4 – 5 times the number of counts for nuclei from non-tumour tissue), one region (region 3) was found in which *EGFR* copy number was only twice normal levels. Note, that due to truncation of nuclei by sectioning, fewer than a diploid number of FISH signals are routinely observed in normal tissue. In the recurrence, amplification of *CCND1* is observed in all regions with only modestly increased copy number of *EGFR* compared to normal tissue

contrast, a similar study using next generation sequencing and microdisscetion of tumour regions could provide genome-wide information on tumour heterogeneity, albeit with much greater computational effort. Nevertheless, there still appears to be an important role for cytogenetic techniques in the analysis of tumours. While the focus here has been on methods to study tumour genomes, techniques such as FISH are compatible with simultaneous analysis of expressed proteins by immunofluorescence [35] and spatial information on intra-tumour genome alterations and cellular phenotypes can be informative with respect to tumour evolution, for example [31].

The variety of cytogenetic and molecular technologies available for measurement of tumour genome alterations provides researchers and clinicians with many choices. Assessment of the differing capabilities, advantages and weaknesses of the technologies should allow selection of the platform best suited to particular applications, including considerations of cost, throughput, sensitivity and resolution.

References

1. Albertson DG, Collins C, McCormick F, Gray JW (2003) Chromosome aberrations in solid tumours. Nat Genet 34:369–376
2. Guan XY, Xu J, Anzick SL, Zhang H, Trent JM et al (1996) Hybrid selection of transcribed sequences from microdissected DNA: isolation of genes within amplified region at 20q11-q13.2 in breast cancer. Cancer Res 56:3446–3450
3. Snijders AM, Hermsen MA, Baughman J, Buffart TE, Huey B et al (2008) Acquired genomic aberrations associated with methotrexate resistance vary with background genomic instability. Gene Chromosome Cancer 47:71–83
4. Savelyeva L, Sagulenko E, Schmitt JG, Schwab M (2006) The neurobeachin gene spans the common fragile site FRA13A. Hum Genet 118:551–558
5. Vogt N, Lefevre SH, Apiou F, Dutrillaux AM, Cor A et al (2004) Molecular structure of double-minute chromosomes bearing amplified copies of the epidermal growth factor receptor gene in gliomas. Proc Natl Acad Sci U S A 101:11368–11373
6. Windle BE, Wahl GM (1992) Molecular dissection of mammalian gene amplification: new mechanistic insights revealed by analyses of very early events. Mutat Res 276:199–224
7. Stephens PJ, Greenman CD, Fu B, Yang F, Bignell GR et al (2011) Massive genomic rearrangement acquired in a single catastrophic event during cancer development. Cell 144:27–40
8. Righolt C, Mai S (2012) Shattered and stitched chromosomes-chromothripsis and chromoanasynthesis-manifestations of a new chromosome crisis? Gene Chromosome Cancer 51:975–981
9. Sorzano CO, Pascual-Montano A, Sanchez de Diego A, Martinez AC, van Wely KH (2013) Chromothripsis: breakage-fusion-bridge over and over again. Cell Cycle 12:2016–2023
10. McClintock B (1942) The fusion of broken ends of chromosomes following nuclear fusion. Proc Natl Acad Sci U S A 28:458–463
11. Birchall PS, Fishpool RM, Albertson DG (1995) Expression patterns of predicted genes from the C. elegans genome sequence visualized by FISH in whole organisms. Nat Genet 11:314–320
12. Speicher MR, Carter NP (2005) The new cytogenetics: blurring the boundaries with molecular biology. Nat Rev Genet 6:782–792
13. Carlson RW, Moench SJ, Hammond ME, Perez EA, Burstein HJ et al (2006) HER2 testing in breast cancer: NCCN Task Force report and recommendations. J Natl Compr Cancer Netw JNCCN 4(Suppl 3):S1–S22; quiz S23-24

14. Gofrit ON, Zorn KC, Silvestre J, Shalhav AL, Zagaja GP et al (2008) The predictive value of multi-targeted fluorescent in-situ hybridization in patients with history of bladder cancer. Urol Oncol 26:246–249
15. Tanas MR, Goldblum JR (2009) Fluorescence in situ hybridization in the diagnosis of soft tissue neoplasms: a review. Adv Anat Pathol 16:383–391
16. van der Burg M, Poulsen TS, Hunger SP, Beverloo HB, Smit EM et al (2004) Split-signal FISH for detection of chromosome aberrations in acute lymphoblastic leukemia. Leuk Off J Leuk Soc Am Leuk Res Fund UK 18:895–908
17. Schrock E, du Manoir S, Veldman T, Schoell B, Wienberg J et al (1996) Multicolor spectral karyotyping of human chromosomes. Science 273:494–497
18. Speicher MR, Gwyn Ballard S, Ward DC (1996) Karyotyping human chromosomes by combinatorial multi-fluor FISH. Nat Genet 12:368–375
19. Liehr T, Starke H, Weise A, Lehrer H, Claussen U (2004) Multicolor FISH probe sets and their applications. Histol Histopathol 19:229–237
20. Kallioniemi A, Kallioniemi OP, Sudar D, Rutovitz D, Gray JW et al (1992) Comparative genomic hybridization for molecular cytogenetic analysis of solid tumours. Science 258:818–821
21. Snijders AM, Nowak N, Segraves R, Blackwood S, Brown N et al (2001) Assembly of microarrays for genome-wide measurement of DNA copy number. Nat Genet 29:263–264
22. Pollack JR, Perou CM, Alizadeh AA, Eisen MB, Pergamenschikov A et al (1999) Genome-wide analysis of DNA copy-number changes using cDNA microarrays. Nat Genet 23:41–46
23. Huang J, Wei W, Zhang J, Liu G, Bignell GR et al (2004) Whole genome DNA copy number changes identified by high density oligonucleotide arrays. Hum Genomics 1:287–299
24. Bignell GR, Huang J, Greshock J, Watt S, Butler A et al (2004) High-resolution analysis of DNA copy number using oligonucleotide microarrays. Genome Res 14:287–295
25. Wang Y, Cottman M, Schiffman JD (2012) Molecular inversion probes: a novel microarray technology and its application in cancer research. Cancer Genet 205:341–355
26. Nilsson M, Malmgren H, Samiotaki M, Kwiatkowski M, Chowdhary BP et al (1994) Padlock probes: circularizing oligonucleotides for localized DNA detection. Science 265:2085–2088
27. Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F et al (2002) Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res 30:e57
28. Homig-Holzel C, Savola S (2012) Multiplex ligation-dependent probe amplification (MLPA) in tumour diagnostics and prognostics. Diagn Mol Pathol Am J Surg Pathol B 21:189–206
29. Geiss GK, Bumgarner RE, Birditt B, Dahl T, Dowidar N et al (2008) Direct multiplexed measurement of gene expression with color-coded probe pairs. Nat Biotechnol 26:317–325
30. Teo SM, Pawitan Y, Ku CS, Chia KS, Salim A (2012) Statistical challenges associated with detecting copy number variations with next-generation sequencing. Bioinformatics 28:2711–2718
31. Zhao M, Wang Q, Jia P, Zhao Z (2013) Computational tools for copy number variation (CNV) detection using next-generation sequencing data: features and perspectives. BMC Bioinform 14(Suppl 11):S1
32. Boeva V, Popova T, Bleakley K, Chiche P, Cappo J et al (2012) Control-FREEC: a tool for assessing copy number and allelic content using next-generation sequencing data. Bioinformatics 28:423–425
33. Alkodsi A, Louhimo R, Hautaniemi S (2014) Comparative analysis of methods for identifying somatic copy number alterations from deep sequencing data. Brief Bioinform 16:242–254
34. Janevski A, Varadan V, Kamalakaran S, Banerjee N, Dimitrova N (2012) Effective normalization for copy number variation detection from whole genome sequencing. BMC Genomics 13(Suppl 6):S16
35. Chuang PT, Albertson DG, Meyer BJ (1994) DPY-27: a chromosome condensation protein homolog that regulates C. elegans dosage compensation through association with the X chromosome. Cell 79:459–474

Chapter 3
Mechanisms of Recurrent Chromosomal Translocations

Richard L. Frock*, Jiazhi Hu*, and Frederick W. Alt

Contents

3.1 Overview: Mechanisms of Recurrent Oncogenic Translocations 28
3.2 Mechanistic Factors Influence Generation of Recurrent Chromosomal Translocations 29
3.3 General Cellular DSBs Provide Translocation Substrates 30
3.4 Programmed DNA DSBs/Rearrangements in Lymphocytes 31
3.5 Involvement of RAG- and AID-Initiated DSBs in Translocations 34
3.6 Role of DNA End-Joining in DSB Repair and Translocations 37
3.7 The ATM DNA Damage Response Pathway and Its Multiple Roles in Suppressing Translocations 39
3.8 Three-Dimensional Genome Organization and Translocations 42
References 44

Abstract Chromosomal translocations frequently involve fusion of the ends of two separate DNA double-stranded breaks (DSBs) at distinct genomic locations. Recurrent chromosomal translocations are found in various cancers. Recently developed high-throughput approaches to clone genome-wide translocations that involve site-specific DSBs have provided new insights into mechanisms of chromosomal translocations. Such studies confirmed that, beyond cellular selection forces, basic mechanistic factors, including DSB frequency and persistence, as well as aspects of three dimensional genome organization, can contribute to recurrent translocations in a cell population. This review discusses our current view of the contribution of such mechanistic factors to recurrent chromosomal translocations.

Keywords Chromosomal translocations • Genomic instability • Lymphocyte development • DNA breaks • DNA repair

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3.1 Overview: Mechanisms of Recurrent Oncogenic Translocations

Chromosomal translocations ("translocations") were observed cytogenetically over 50 years ago; since then, recurrent translocations have been identified in many types of cancers, most frequently in lymphoid and myeloid neoplasms, but also in solid tumours, such as lung and prostate cancers [1–4]. Translocations can contribute to both initiation and progression of neoplastic transformation, most frequently by leading to abnormal activation of cellular oncogenes [3]. In this regard, many translocations likely arise at very low frequency and are strongly selected during the tumourigenesis process leading to their appearance as clonal events in tumour cells. Cancer genome studies indicate that most translocations result from end-joining of two DNA double-stranded breaks (DSBs) that occur at two separate genomic locations. In this context, most joins of DSB ends from two distinct DSBs, whether on a separate chromosome to generate translocations or on the same chromosome to generate interstitial deletions or inversions, appear mechanistically related and can be considered as translocations [5].

Translocations between two genes can result in expression of hybrid fusion proteins, which generate aberrant activation of proto-oncogenes. A notable example is the *BCR-ABL1* translocation between human chromosomes 9 and 22, also known as the Philadelphia chromosome, that is found in chronic myelogenous leukaemia and early B cell leukaemias [6–8]. Many other such examples for other tumour types have been elucidated [9, 10]. Translocations between different genomic sequences also can activate proto-oncogenes by deregulating their expression, often by linking them to strong *cis*-regulatory elements, a mechanism common in lymphoid malignancies in which translocations link strong transcriptional enhancers or super-enhancers in antigen receptor loci to cellular oncogenes [4, 9, 11]. A classic example of this type of oncogenic translocation are translocations that fuse immunoglobulin (IG) heavy chain locus (*IGH*) and its 3′ regulatory region, a known super-enhancer, to the *MYC* oncogene in Burkitt lymphomas (BL), with a variety of others having been well-characterized [2, 4].

Oncogene overexpression in tumours also can be achieved via gene amplification, the first form of genomic instability in cancer cells described at the molecular level [12]. One mechanism for oncogene amplification in cancer models involves generation of dicentric chromosomal translocations with breakpoint fusions in the vicinity of oncogenes [13–16]. In cancer cells deficient in the cellular G1 DSB checkpoint (e.g., TP53 or ataxia telangiectasia mutated (ATM) protein deficient), breakage-fusion-bridge cycles [17] (BFB) of such dicentrics can rapidly lead to oncogene amplification [18]. Although such BFB mechanisms of oncogene amplification are likely most common during solid tumour progression [15, 19, 20], they have also been observed in human B cell malignancies [21], including multiple myeloma [22]. In ATM-deficient mouse T cell lymphoma models, dicentric chromosomes that result from aberrant V(D)J recombination events at T cell receptor (TCR) δ loci (*TRD*) lead to BFB-generated amplification of linked sequences and to new BFB-generated DSBs that participate in translocations that may delete tumour

suppressor genes [23] (see below). Inter- and intra-chromosomal translocations/deletions have been implicated in deletions of tumour suppressors that contribute to various cancers [24–27].

3.2 Mechanistic Factors Influence Generation of Recurrent Chromosomal Translocations

Beyond cellular selection for oncogenic translocations, general mechanistic factors of translocations can affect the propensity of two genomic sequences to translocate to each other recurrently, and can even impact oncogene choice in different malignancies [28, 29]. Many recent studies of mechanistic factors involved in promoting translocations have been done in lymphoid cells, which will be a main focus of this review.

As translocations often involve the fusion of ends from two separate DSBs, the frequency of DSBs at the two participating sites to be joined will directly influence the rate of a particular translocation. In this context, such DSB frequency will reflect both the frequency at which the participating DSBs are generated by various mechanisms and also how long they persist before being properly repaired [5] (see below). In the latter context, DSB persistence reflects the efficiency at which DSBs are repaired. Mammalian cells possess multiple DSB sensing mechanisms that are linked to DSB repair pathways that efficiently repair DSBs, often by end-joining them back together [30–32]. DSB sensing pathways, in addition to participating in repair, also activate checkpoints that either delay cell cycle progression of cells with unrepaired DSBs until they are joined or eliminate cells with persistent unjoined DSBs [30, 33] (see below). The two major pathways for DSB repair are homologous recombination (HR) [34], which is primarily involved in repair of post-replicative DSBs, and classical non-homologous DNA end-joining (C-NHEJ) which is functional throughout the cell cycle but is predominant in G1 when HR is not active [35] (discussed below). We will focus mainly on C-NHEJ due to the exclusive involvement of this repair pathway in joining programmed DSBs in lymphocytes and in suppressing their translocation [35].

For two separate DSBs to be joined to form translocations, they must also be juxtaposed ("synapsed") at the time they are broken [5]. Thus, in a population of cells, the synapsis frequency of two regions that contain DSBs will directly influence their translocation frequency. Certain sequences are more frequently synapsed than others in the genome due to general principles of chromatin folding, as well as being involved in common processes, such as transcription [36, 37]. Beyond this, translocation of DSBs that are much less frequently synapsed in cells within a population can still occur, due to cellular heterogeneity with respect to three-dimensional (3D) spatial genome organization [38]. At a local level, synapsis of breaks that are in relatively close proximity also may occur through Brownian or Langevin motion [38–40]. Finally, active movement of DSB ends has been reported in yeast [41, 42] and in mammalian cells [43, 44], and may also contribute to synapsis.

In the past, inability to identify newly occurring translocations in normal cells, without the biases imposed by cellular or oncogenic selection, has limited analyses of mechanistic factors involved in the generation of translocations. Such limitations have been overcome by the recent development of high-throughput genome-wide translocation cloning techniques [45, 46]. In this regard, the use of rare-cutting, site-specific restriction endonucleases, such as the yeast I-*Sce*I meganuclease or homothallic (HO) endonuclease, have provided critical tools for studying translocations by providing a method to generate initiating DSBs at one or more desired genomic locations [47]. The finding that I-*Sce*I generated DSBs at introduced target sites in the *IgH* locus in B lymphocytes could substitute for endogenous mechanisms that generate *IgH* class switch recombination (CSR) provided the foundation for the development of genome-wide methods for studying translocation mechanisms [48]. These high-throughput methods, referred to generically here as "translocation cloning" methods, have been used to identify endogenous DSBs genome-wide based on their translocation to fixed I-*Sce*I induced "bait" break-sites in activated B lymphocytes or G1-arrested progenitor B cells lines as well as in non-lymphoid cells [38, 39, 45, 46, 49].

Advances in custom nuclease tools that target endogenous genomic sequences, including zinc finger nucleases (ZFNs) [50], transcription activator-like effector nucleases (TALENs) [51], and Cas9:gRNAs [52], have provided additional ectopic DSB-generating approaches to study generation of translocations from DSBs at specific sites in mammalian genomes without introducing a target DSB sequence [39, 53, 54]. Recently, Cas9:gRNAs and TALENs have been used to very successfully generate bait DSBs for translocation cloning at desired endogenous sites in human cells [55], allowing tests of basic principles of translocations, modelling of recurrent oncogenic translocations, and potentially the development of improved cancer diagnostics. This approach has also provided potential benefits to genome-engineering and gene therapy fields by providing a very robust method to identify genome-wide off-target and wide-spread, low-level DSB activity of custom nucleases and to also detect collateral damage of such agents including recurrent translocations and/or deletions [55].

Below, we will further discuss the mechanistic factors outlined above and how they can contribute to recurrent translocations or recurrent classes of translocations based on insights obtained from the recently developed translocation cloning assays. We will focus this discussion largely on mechanisms revealed from studies of lymphoid cells and tumour models, but also indicate the more general relevance of the findings of these studies.

3.3 General Cellular DSBs Provide Translocation Substrates

Human dividing cells may undergo as many as 50 DSBs per cell cycle [56]. General DSBs can occur spontaneously or be induced by various endogenous or exogenous damaging factors. Some byproducts generated during cell metabolism, including

reactive oxygen species and endogenous alkylating agents can lead to DSBs [57]. DNA replication is another source of endogenous DSBs [58]. When encountering DNA lesions or other replication barriers, DNA replication forks stall, accumulate single-stranded DNA (ssDNA) and finally collapse, resulting in DSBs [59–61]. Fragile sites, a common replication barrier, provide break-sites for many gross chromosomal rearrangements found in early-stage tumours or precancerous cells [58]. Fragile sites appear to contribute to breakpoints of recurrent translocations in acute lymphoblastic, myeloid leukaemias, or BL (See Chap. 5 by Jiang et al., "Common Chromosomal Fragile Sites and Cancer") [62–64]. Recent studies have also identified another class of "early replicating fragile sites" in B lymphocytes that were enriched in areas of repetitive sequences and/or CpG nucleotides, some of which map near translocation breakpoints in B cell lymphomas [65]. Transcription also has been implicated in DSB generation [36, 45, 46, 66]. Transcription-associated DSBs may result from head-on collisions between DNA and RNA polymerases [67, 68], topological constraints arising from transcription induction [69], or formation of unstable DNA structures, such as R-loop and G-quadruplexes [70–72]. Transcription has also been implicated in the generation of DSBs that participate in translocations in activated B lymphocytes [45, 46] (see below).

In addition to endogenous factors, DSBs also can be generated by exposure to external agents, such as ionizing radiation (IR) and various types of chemotherapeutics [73, 74]. 1 Gray (Gy) of γ-irradiation generates about 20 DSBs in mammalian cells [75]. Translocation cloning studies from IR-treated cells confirmed that IR-derived non-specific DSBs can generate translocation substrates genome-wide [38]. Topoisomerase II inhibitors, commonly used for anticancer treatment, prevent topoisomerase II from releasing topological constraints during DNA replication or transcription, thus promoting DSBs, implicated, for example, in the emergence of therapy-related myeloid neoplasms with recurrent oncogenic translocations [76, 77].

3.4 Programmed DNA DSBs/Rearrangements in Lymphocytes

DSBs are necessary intermediates of the programmed rearrangements that take place during the V(D)J recombination process that assembles diverse sets of antigen receptor gene segments in developing B and T lymphocytes and the CSR process that changes the expressed IGH constant region (C_H) exons in activated mature B lymphocytes [5]. These programmed DSB-based gene rearrangement processes, which might be considered programmed intra-chromosomal translocations, involve the coordinated introduction of two separate DSBs at targeted *IGH* locus sites followed by their joining [35, 78]. Although the joining of these DSBs is generally regulated to ensure "proper" joining within *IGH*, they also can be aberrantly joined to other genomic DSBs to generate oncogenic translocations [5, 79].

The B cell receptor (BCR) is composed of two pairs of identical IGH and IG light chains (IGL/IGK). The secreted forms of BCRs are known as antibodies. Similarly, TCRs are heterodimers of either αβ or γδ chains [80, 81]. The exons that encode the N-terminal antigen-binding variable region exons of IGH chains are somatically assembled in progenitor B lymphocytes from 100s of different variable (V), 13 diversity (D), and 4 joining (J) genes that lie within distinct segments of the several megabase (Mb) long variable region portion of the *IgH* locus [5] (Fig. 3.1). Organization of *IgL*, *IgK* and *TcR* loci is similar and the general aspects of cleavage and joining of *IgH* V, D, and J segments outlined below also applies to these loci. The lymphocyte-specific endonuclease, RAG endonuclease, comprised of the recombination activating gene 1 and 2 proteins (RAG1/RAG2) [82] initiates V(D)J recombination by introducing DSBs between appropriate pairs of V, D, or J coding segments and short conserved recombination signal sequences (RSSs) that flank them; notably, RAG does not cleave, at least efficiently, at isolated RSSs [83]. RAG cleavage generates a pair of hairpin-sealed coding ends and a pair of blunt, broken RSS ends [81, 84].

The D and J segments lie proximal to each other in *IgH* and are cut and joined first in development. Subsequent, synapsis of V_H segments with the DJ_H complex is thought to be facilitated by physical contraction of the *IgH* locus [85, 86], which enhances synapsis by bringing the 100s of V_H segments scattered over Mb linear distances into close enough proximity to sample the DJ_H complex by a form of diffusion referred to as Langevin motion [40]. The RAG complex also binds DJ_H regions, facilitated by particular histone modifications associated with transcription [87–90] before V_H synapsis allowing formation of "recombination centres" that stabilize synapsed V_H to DJ_H complexes once formed and which generate paired RSS cleavage [90]. Formation of D_H to J_H joins likely occurs similarly, but due to the close proximity of the D_H and J_H segments, may not require physical locus contraction. Following cleavage, RAG, with the aid of other repair factors (see below), holds coding and RSS ends in a post-cleavage synaptic complex [84], and channels their repair exclusively to the C-NHEJ pathway [5, 91], C-NHEJ directly fuses the blunt RSS ends and, along with other factors, further processes coding ends before joining them, thereby contributing to V(D)J exon coding diversity [5]. The RAG post-cleavage complex, and perhaps other factors, also contributes to directing the joining of coding ends to each other and RSS ends to each other, thereby prescribing a specific chromosomal orientation of V(D)J recombination which results in deletions or inversions depending on the orientation of the participating V, D, and J segments [5, 92].

The portion of the *IgH* locus downstream of the V, D, and J segments contains multiple sets of exons encoding for different C_Hs within an approximately 200 kb region [93, 94]. The Cμ exons, which lie closest to the V(D)J, are transcribed to yield a V(D)J Cμ transcript that encodes μ heavy chains, which activates assembly of IgL/IgK variable region exons and ultimately associate with IgL/IgK chains to form an IgM BCR resulting in "mature" B lymphocytes [95]. Antigen-dependent activation can induce mature B cells to undergo CSR to exchange Cμ exon for one of the sets of C_H exons that lie downstream (Fig. 3.1). CSR involves introduction of DSBs into a donor switch (S) region just upstream of Cμ and into an acceptor S region upstream of a targeted set of downstream C_H exons. Subsequently, the

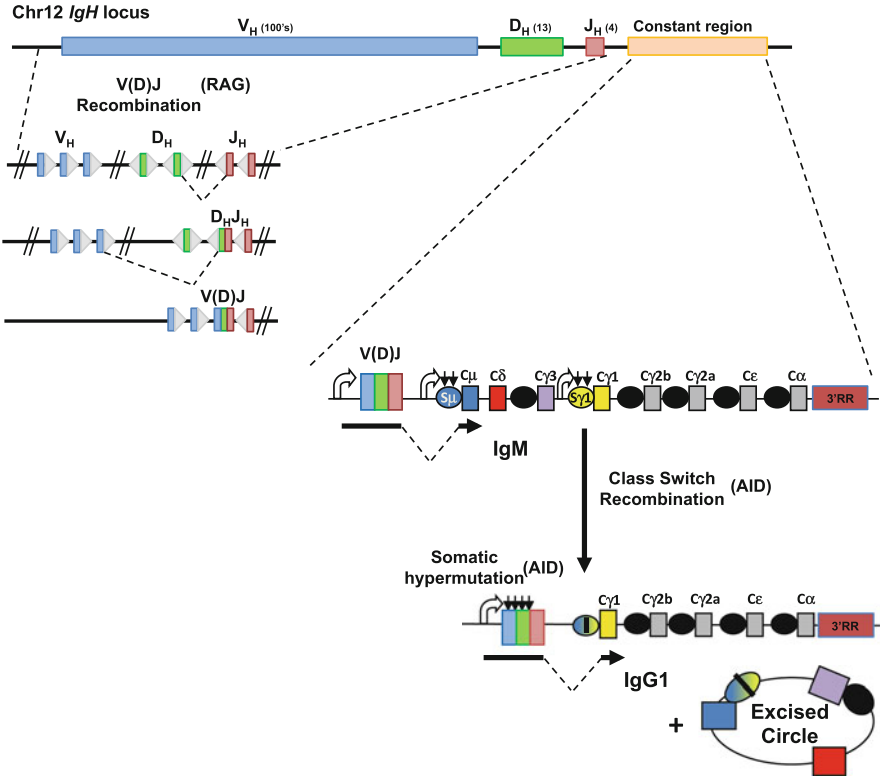

Fig. 3.1 Programmed DSBs and genomic rearrangements in developing and mature B cells. (*Top*) Diagram of the murine *IgH* locus on chromosome 12 (not to scale). The *IgH* locus contains of hundreds of V, 13 D, and 4 J gene segments arranged in clusters as indicated. (*Left*) In pro-B cells, RAG cleaves synapsed V, D, and J gene segments at appropriately paired RSSs (*grey triangles*) which are then joined by C-NHEJ factors to generate V(D)J exons (see text for details). In the *IgH* locus, DJ_H joining occurs first followed by V_H gene segment joining to the DJ_H segment. See text for more details. (*Right*) In response to antigen, mature B cells can undergo CSR to exchange their initially expressed *IgH* C_H exons from Cμ to one of a set of downstream (from 100 to 200 kb) exons encoding Cγ3, Cγ1, Cγ2b, Cγ2a, Cε, or Cα. Each set of C_H exons is preceded by a long (1–10 kb) repetitive Switch (S) sequence (*ovals*). Transcription through the donor Sμ and a target downstream S region promotes AID-initiated DSBs (*arrow heads*) which are then joined between donor and acceptor S regions to delete the intervening sequences and replace Cμ with the targeted downstream C_H (See text for more details). AID can also introduce somatic mutations into the assembled V(D)J to allow affinity maturation of the BCR (See text for more details)

upstream end of the DSB in Sμ is joined to the downstream end of the DSB in the target S region to delete Cμ and other intervening sequence and juxtapose the new set of C_H exons to the V(D)J exon [94]. Exchanging the C_H exon, changes the effector functions of the expressed antibody. Unlike V(D)J recombination, which is completely dependent on C-NHEJ, joining of DSBs within S regions to complete CSR can occur, at somewhat reduced levels, in the absence of C-NHEJ via alternative end-joining (A-EJ) pathways [35] (see below).

CSR is initiated by the activation-induced cytidine deaminase (AID) encoded by the AICDA gene [96], which acts on ssDNA to deaminate cytidine residues within short (4 bp) target motifs of which the sequence AGCT is a canonical representative [97, 98]. S regions are very long (1–10 kb) and very rich in AID target motifs [99]. AID cytidine deamination also initiates an antigen dependent variable region diversification process termed somatic hypermutation (SHM) [100]. During CSR and SHM, AID-initiated C to U lesions are processed into DSBs and point mutations, respectively, via related processes that require activities of the normal base excision and mismatch repair pathways [97, 101]. Various mechanisms have been proposed to promote DSB versus mutational processing of AID, although these two outcomes are not totally separable [93, 100–102]. Cytidine deamination of target sequences by AID requires their transcription to both recruit AID and provide ssDNA substrates [5]. Following transcription of the GC-rich S regions, AID access to the non-template strand is promoted by the formation of stable R-loops [71, 72]. Additional details of mechanisms of AID targeting to *IgH* S regions and variable regions exons also have been elucidated [5, 103–105].

Currently, S region synapsis has been proposed to potentially involve diffusion (Langevin motion) due to their relatively proximal location within a 200 kb domain, with high levels of AID-initiated DSBs helping to ensure breakage of S regions while synapsed [39] (see below). Aspects of *IgH* locus organization, and potentially the 53BP1 (TP53BP1) DSB response factor (see below), may also help to facilitate/stabilize S region synapsis [103, 106]. Unlike RAG-initiated DSBs, S region DSBs can occur in unsynapsed S regions; however, these DSBs are usually joined internally in S regions to generate intra-S region deletions as opposed to translocation [93]. As AID, thus far, has no known downstream roles in S region synapsis, and indeed, substantial CSR can be generated by I-*Sce*I-initiated DSBs at target sites replacing S regions [39, 48], CSR may be analogous to a targeted form of an intrachromosomal translocation [48]. CSR must occur in a deletional, versus inversional, orientation to generate productive CSR; however, while RAG cleavage and generation of a post-cleavage complex may contribute to orientation-specific joining during V(D)J recombination, little has been reported about if and how orientation-specific joining occurs during CSR [5]. If joining is orientation specific during CSR, it must employ specialized mechanisms since, in translocation cloning assays, bait DSBs generally join equally to both ends of other DSBs across the genome in activated B lymphocytes [45].

3.5 Involvement of RAG- and AID-Initiated DSBs in Translocations

The potential of RAG-initiated DSBs to contribute to translocations is counteracted at several levels. RAG cleavage is restricted by the "12/23 rule" to paired RSSs with appropriate complementarity, which limits generation of "off-target" RAG-cleavage at "cryptic" RSSs across the genome [84]. RAG expression also is limited to

G1-phase lymphoid cells [87], which contributes to restricting repair to C-NHEJ and also provides the G1 checkpoint to prevent replicative propagation of RAG initiated DSBs, for example via dicentric formation [13, 16, 49]. Moreover, formation of the RAG post-cleavage synaptic complex limits direct joining of coding and RSS ends and limits availability to translocate to other DSBs. Finally, the ATM DSB response complex [107] cooperates with RAG2 [108, 109] to stabilize of RAG-initiated post cleavage DSB complexes and to prevent their separation and translocation (see below). In the latter context, translocation cloning from G1-arrested ATM-deficient pro-B cell lines that induce RAG, clearly showed I-*Sce*I or RAG-induced bait DSBs can translocate to other DSBs genome-wide, but endogenous hotspots were all provided by RAG-initiated DSBs at various *Ig* and *TcR* loci (due to the high frequency of target DSBs in these loci) and, in IR-treated cells, to DSBs genome-wide with highly preferential joining to DSBs *in cis* on the same chromosome due to 3D proximity influences [38] (Fig. 3.2). In this context, oncogenic translocations between V(D)J recombination-associated DSBs and other DSBs have been demonstrated to occur in the context of TP53/C-NHEJ deficient mouse pro-B cell lymphoma models [13, 16].

Translocations involving RAG-initiated DSBs at *TCR* loci, with translocations from *TRD* segments being most prominent, and DSBs near various oncogenes, including *TAL1* and *TAL2*, *LMO1* and *LMO2*, and *MYC*, are common in human T-cell acute lymphoblastic leukaemia (T-ALL) [27]. Among human B cell tumours, translocations involving *IG* variable region gene segments are rare in B-ALL, accounting for only about 3 % of cases [110], but are found in mature B cell neoplasms, such as the recurrent *IGH*/*MYC* translocation in endemic BL [79] or *IGH*/*BCL2* translocation in follicular lymphomas [2]. Mouse model studies suggest some such translocations may persist through development [111] (see below) or result from RAG activity during secondary recombination events in peripheral B cells referred to as receptor editing [29, 112]. RAG can also contribute to oncogenic translocations by generating DSBs at RSS-like sequences across the genome, termed cryptic RSSs (cRSSs); the frequency of cRSSs in the human genome has been estimated at about 1 per 500 bp [113, 114]. A recent survey of a large series of translocation junctions between *TCR* loci and various oncogenes in human T-ALL revealed that about 25 % of such rearrangements involved cRSSs at the TCR translocation partner loci [27]. In addition, some interstitial deletions that contribute to human T-ALL oncogenesis appear to involve RAG-initiated DSBs at cRSSs in both TCR and/or non-TCR partners [27, 115]. Finally, recent studies in ATM-deficient mouse B cell lymphomas also suggested that oncogenic translocations originated from RAG cutting at *IgH* loci and putative cRSSs downstream of *Myc* [111].

AID-generated DSBs in *IGH* S regions during CSR have been implicated in the generation of recurrent oncogenic translocations in human mature B cell lymphomas involving *IGH* and AID off-targets, including the *IGH*/*BCL6* translocation in diffuse large B-cell lymphoma [116] and *IGH*/*MYC* translocations in sporadic BL [79]. AID-initiated DSBs in V(D)J exons during SHM also have been implicated in certain oncogenic translocations in human B cell lymphomas [117]. Translocation cloning studies in activated mouse B cells clearly demonstrated that, beyond the

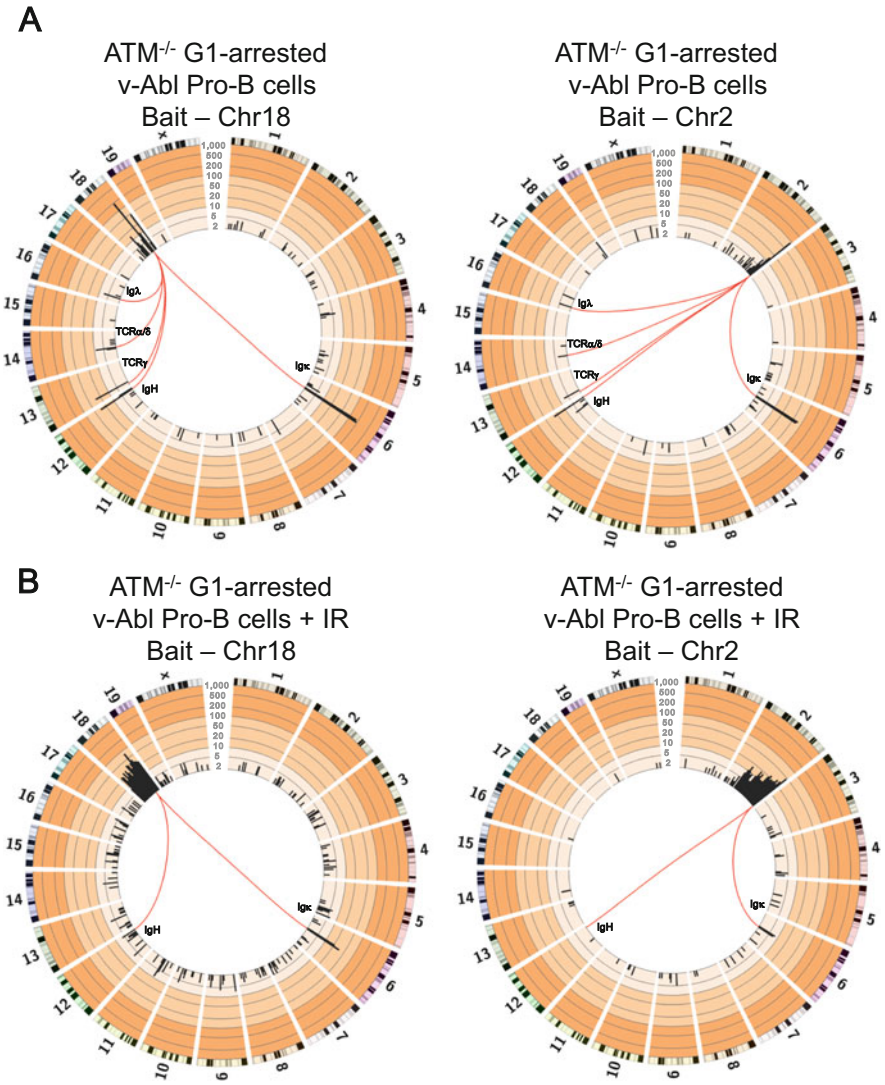

Fig. 3.2 Circos plots of G1-arrested pro-B translocation cloning libraries. (**a**) custom circos plots (See ref. [55]) of genome-wide translocation cloning libraries from two different G1-arrested pro-B cell lines with singly integrated I-*Sce*I substrate bait DSB sequence on either chromosome 18 (*left*) or chromosome 2 (*right*). Both RAG and I-*Sce*I were induced to generate endogenous DSBs in the G1-arrested cells prior to genome-wide library generation from the I-*Sce*I bait DSB. Black bars indicate I-*Sce*I translocation junction frequency to genome-wide DSBs over 5 Mb bins on a custom log scale plot. Inner *red lines* link the bait DSB site to recurrently joined antigen receptor loci (e.g., show translocation hotspots). In these cells, all translocation hotspots from either chromosome 18 or chromosome 2 baits were RAG-initiated DSBs at the various endogenous antigen receptor loci. No other translocation junction regions qualified as hotspots. (**b**) IR treatment of the same pro-B lines to introduce frequent DSBs genome-wide (e.g., normalize DSB frequency genome-wide) decreased enrichment of antigen receptor locus translocation junctions and led the endogenous *cis* chromosome containing the bait sequence to become a translocation hotspot region (due to increased influence of 3D proximity when DSBs are not limiting) In these plots, all libraries are size-normalized to allow direct comparison (See ref. [55]). Chromosomes are displayed centromere to telomere in a clock-wise orientation (Data are adapted from [38]. See text, ref. [5, 38] for further details)

IgH locus S region targets, AID activity also promotes lower level DSBs at dozens of other genes across the genome, referred to as AID off-targets [45, 46]. These studies also demonstrated that both *IgH* and off-target DSBs translocated robustly to bait I-*Sce*I-generated DSBs in the *Myc* locus [45, 46, 49, 118].

IGH S regions and IG V(D)J exons likely have evolved mechanisms to recruit AID activity during CSR and SHM, such as high density AID target motifs and transcription dependent ability to generated secondary structures such as R loops for S regions [93, 119]. However, how AID is directed to off-target sites in activated B cells is still under investigation. In this context, AID is also directed to a series of off-target sites in germinal centre B cells that may contribute to SHMs and occasional DSBs that contribute to germinal centre B cell lymphomas [10, 120, 121]. Translocation cloning studies in CSR-activated B cells demonstrated high correlations between AID-dependent translocation hotspots and active transcription start sites, with translocations often clustering just downstream of active TSSs [45, 46]. However, as the vast majority of transcribed genes in activated B cells are not AID off-targets, additional factors beyond transcription per se must be involved in such AID "off-targeting" [5]. Recent studies have demonstrated that such factors include "super-enhancers" and convergent transcription [122].

3.6 Role of DNA End-Joining in DSB Repair and Translocations

The C-NHEJ machinery comprises four evolutionarily conserved "core" factors, Ku70 (XRCC6), Ku80 (XRCC5), XRCC4 and DNA ligase 4 (LIG4), which are essential for joining all types of DSBs via C-NHEJ [35]. Two additional C-NHEJ factors include DNA-dependent protein kinase catalytic subunit (PRKDC/DNA-PKcs) and the Artemis endonuclease (DCLRE1C), which together are important for joining DSBs in need of further processing, such as opening hairpin V(D)J coding ends [123]. The XRCC6/XRCC5 (Ku) heterodimer provides the C-NHEJ DSB recognition component, which binds DSBs to protect them from resection [124] and recruits downstream factors including PRKDC (to form the DNA-PK holoenzyme). Ku also recruits XRCC4 and LIG4, which form the ligase complex for C-NHEJ [35]. All of these factors are required for generation of V(D)J recombination coding joins; with the core factors being absolutely required for both coding and RSS joins [35, 125]. The XLF (NHEJ1) ("XRCC4 like factor") also has been implicated in C-NHEJ based on the IR sensitivity and apparent DSB repair defects in human patients with *NHEJ1* mutations [126, 127]. XLF, which interacts with the XRCC4-LIG4 complex [128–130], has been suggested to play a role in ligation of DSBs with incompatible or blunt ends [131] (see below). Deficiency of any core C-NHEJ factors dramatically increases genome instability, including translocations, in various cell types; whereas deficiency for the other C-NHEJ factors increases genomic instability but usually not as dramatically [35, 125]. In mice, deficiency for core C-NHEJ factors and DCLRE1C and PRKDC leads to severe combined

immunodeficiency ("SCID") due to inability join V(D)J recombination-associated breaks required for assembly of antigen receptor genes [5]. LIG4 hypomorphic mutations and XLF deficiencies also lead to variable immunodeficiency due to V(D)J recombination defects in human patients [132, 133].

Mice deficient for XRCC4 and LIG4 die in late embryonic development in association with severe apoptosis of newly developed neurons, along with abrogated V(D)J recombination [134, 135]. Less severe neuronal apoptosis occurs in Ku-deficient mice [136]. When XRCC4 or LIG4-deficiency are combined with TP53-deficiency, which removes the TP53-dependent G1 DSB checkpoint, neuronal apoptosis and embryonic lethality (but not V(D)J recombination) are rescued [137, 138]. Notably, TP53-deficient mice that are also deficient for any core C-NHEJ factor or for PRKDC or DCLRE1C die from progenitor B cell lymphomas that generate RAG-dependent dicentric translocations between the *IgH* locus and the *Myc* (or *N-myc*) loci leading to amplification of these oncogenes [9, 139]. The generation of such dicentric translocations and BFB cycles results from propagation of the RAG-generated *IgH* locus breaks through the cell cycle in the absence of the G1 DSB checkpoint enforced by TP53 [16]. Where examined, such C-NHEJ and TP53 double-deficient mice also develop medulloblastomas in situ and, indeed, conditional inactivation of XRCC4 in developing neurons of TP53 deficient mice leads to inevitable medulloblastomas with highly recurrent translocations and gene amplifications which include *N-myc* or *Myc* amplifications [140, 141]. Why C-NHEJ is required for neural development and protection from medulloblastomas with recurrent translocations is not yet known. Notably, XLF plus TP53 double deficient mice do not generally succumb to B lineage lymphomas, reflecting lack of absolute requirement for XLF in V(D)J recombination in an otherwise normal background (see below); but they do develop medulloblastomas, reflecting the requirement for XLF in general DSB repair by C-NHEJ [142].

The frequency of translocations that form in various types of core C-NHEJ deficient cells [141, 143–145] and the recurrent translocations that occur in core C-NHEJ-deficient tumours revealed that chromosomal translocations can be catalyzed by A-EJ pathways [16, 35]. In this regard, C-NHEJ-deficient mammalian cells join DSBs in plasmid-based assays by A-EJ pathway [146, 147]. Likewise, while V(D)J recombination absolutely requires C-NHEJ, CSR can occur at up to 50 % normal levels in the absence of core C-NHEJ factors [148, 149], or even the absence of both Ku70 plus LIG4—which eliminates both DSB recognition and joining components of C-NHEJ [148]. The latter studies definitively prove the existence of relatively robust A-EJ pathways in mammalian cells that are completely distinct from C-NHEJ. A number of known DNA repair factors have been implicated in A-EJ pathways (reviewed by [35]).

CSR junctions in the different types of C-NHEJ deficient cells were essentially totally (e.g., XRCC4 or LiG4-deficiency), or substantially (e.g., Ku-deficiency), mediated by short micro-homologies (MHs) [148, 149]. Similarly, oncogenic translocation junctions found in nine independent XRCC4- or LIG4- plus TP53-deficient pro-B lymphomas were MH-mediated [16]. In this regard, short MHs are found in many translocations genome-wide in activated mouse B cells [45], human

cancer genomes [150], and tumour translocation junctions [151]. However, A-EJ can also generate substantial levels of blunt junctions in various contexts, perhaps dependent on the DSB ends presented for joining [152, 153]. Moreover, C-NHEJ frequently uses short MHs [154, 155]. Therefore, A-EJ, which could represent several different pathways [35], cannot be categorized unequivocally as MH-mediated. It should also be noted that, while A-EJ may, indeed, be a translocation prone pathway [156], its predominant contributions to translocations in the absence of C-NHEJ also may be contributed to increased levels of unrepaired substrate DSB ends for translocations [32]. Finally, C-NHEJ may contribute to translocations in C-NHEJ proficient cells (e.g., [157]); although the relative contribution of A-EJ remains to be determined.

3.7 The ATM DNA Damage Response Pathway and Its Multiple Roles in Suppressing Translocations

Ataxia telangiectasia (AT), a syndrome characterized by neurodegeneration, immunodeficiency, sensitivity to ionizing irradiation, and cancer susceptibility is associated with mutations in the *ATM* gene [158]. In response to DSBs, ATM, a serine/threonine kinase, activates a downstream DNA damage response (DDR) pathway that includes series of chromatin bound factors that regulate cell cycle progression at the G1 checkpoint and contribute directly to DSB repair by C-NHEJ [5, 31]. A key ATM substrate is the TP53 tumour suppressor, a transcription factor that directly activates the G1/S cell cycle checkpoint to arrest cells for DSB repair or that triggers apoptosis to eliminate cells with persistent DSBs [159]. ATM DDR substrates include the H2AX (H2AFX) histone variant, MDC1, and 53BP1, which assemble into large macromolecular complexes, called "foci", that can spread in chromatin over several hundred kb or more on either side of DSBs [160, 161]. The DDR also employs additional downstream factors that facilitate repair pathway choice and provide additional chromatin modifications that promote DSB repair (reviewed by [34, 162–165]).

The ATM DDR has been implicated in contributing directly to C-NHEJ of DSBs, potentially by tethering DSB ends and, thereby, contributing to appropriate rejoining by C-NHEJ [166, 167]. In this regard, ATM deficiency has long been known to lead to genomic instability and recurrent translocations, particularly in lymphoid cells and tumours [168, 169]. Such translocations are likely facilitated by the dual effects of ATM deficiency on C-NHEJ (e.g. during V(D)J recombination) and abrogation of G1 DSB checkpoint [78], analogous to combined C-NHEJ and TP53 deficiency. Recent translocation cloning studies have confirmed the increased levels of genome-wide translocations from DSBs in the *Myc* gene in ATM-deficient activated mouse B cells relative to wild-type B cells [49] (Fig. 3.3).

Like ATM deficiency, H2AX deficiency in various cell types leads to marked increases in genomic instability, increased chromosomal translocations [170, 171] and, in the absence of TP53, progenitor and mature B cell lymphomas with complex

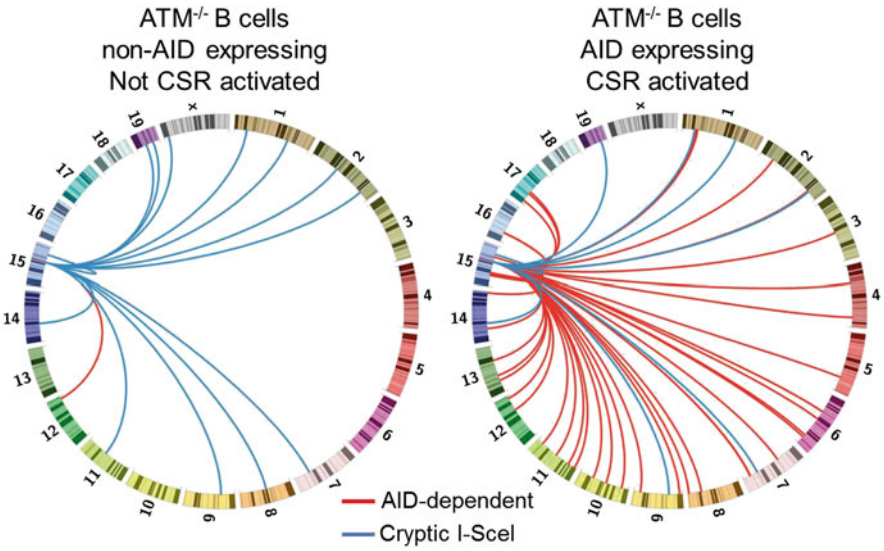

Fig. 3.3 Circos plots of stimulated primary B cell high throughput genome-wide translocation sequencing libraries. Translocation libraries generated with a bait I-*Sce*I break-site in intron 1 of *Myc* gene on chromosome 15 from CSR-activated primary B cells that either do not express AID (*left* panel) or do express AID (*right* panel). *Blue* and *red lines* link the *Myc* I-*Sce*I bait DSBs to cryptic I-*Sce*I-generated translocation hotspots genome-wide and *red lines* link bait DSBs to AID-dependent hotspots genome-wide. Chromosomes are displayed centromere-to-telomere in a clockwise orientation (Data are adapted from [49]. See text and ref. [49] or further details)

Myc translocations (involving *IgH*) and amplifications [172–174]. ATM or H2AX deficiency also moderately impairs CSR (decreasing levels to about 50 % or less of normal [5, 160]) accompanied by accumulation of substantial levels of AID-dependent *IgH* locus chromosome breaks and translocations [171, 174]. Notably, however, 53BP1 deficiency, while not dramatically increasing genomic instability in most tested cell types other than CSR-activated B cells, nearly abrogates CSR [175, 176]. Yet, 53BP1 deficiency leads to similar level of AID-dependent *IgH* breaks and translocations as observed in the context of ATM- or H2AX deficiency, which together with other findings suggest a specialized role for 53BP1 in CSR that may involve S region synapsis, end-protection, or other yet to be identified functions [103, 125].

Deficiency for ATM also has moderate effects on V(D)J recombination that have been attributed to destabilization of the post-cleavage synaptic complex, allowing some RSS or coding ends to escape and participate in translocations [78]. Correspondingly, high-throughput translocation libraries from ATM-deficient pro-B cell lines revealed the major translocation hotspots from various bait DSBs in different chromosomal locations to be the various *Ig* and *TcR* loci which are RAG-targets in these cells [38] (Fig. 3.2). Despite the impact on CSR, C-NHEJ and

genomic stability in activated B cells, deficiencies for downstream ATM substrates H2AX and 53BP1 have little or no obvious effect on V(D)J recombination [170, 176, 177]. The relatively modest impact of deficiencies of ATM DDR factors on V(D)J recombination results from functional redundancy between these factors with the small XLF factor [178]. In this regard, despite the C-NHEJ role implied by the phenotype of XLF-deficient human patients and their cells, XLF deficiency in mice does not markedly impact V(D)J recombination in developing lymphocytes, despite leading to more general genomic instability and IR sensitivity [142]. However, combined deficiency for XLF and ATM, H2AX or 53BP1 leads to an essentially complete block in V(D)J recombination, along with more general DSB repair and CSR defects that indicate a nearly complete loss of C-NHEJ [178–180] (reviewed by [125]). Thus, in the absence of XLF, ATM and downstream DDR factors are required for C-NHEJ and vice versa, raising the possibility that variations in the expression of XLF in different tissues or individuals could contribute to differential manifestations of ATM deficiency. The nature of this functional redundancy is still being studied [125].

In humans, germline or somatic mutations in *ATM* have been associated with development of both B and T cell lymphomas [181, 182]. However, ATM deficiency in mice predisposes only to thymic lymphomas, but not B cell lymphomas [168]. ATM-deficient T cell lymphomas nearly universally have complex translocations involving the *Trd* (*Tcrd*) locus on chromosome 14 [23]. Notably, *TRD* translocations are the most common oncogenic translocation in human T-ALLs [27]. In mouse T cell lymphomas, the translocations involve formation of dicentric chromosomes downstream of RAG-induced *Trd* DSBs and subsequent amplification of chromosome 14 sequences along with potential *Trd* translocation-mediated deletion of a tumour suppressor on chromosome 12 [23]. Notably, TP53-deficient mice that harbour a homozygous germline mutation that leads to a C-terminal truncation in the RAG2 protein develop T cell lymphomas with essentially identical *Trd*-based translocations as observed in ATM deficient T cell lymphomas [109]. In this case, the RAG2 truncation is speculated to destabilize the post-cleavage V(D)J recombination complex similar to ATM deficiency [109].

Recently, several mouse models have been generated that develop peripheral mature B cell lymphomas in the context of ATM-deficiency [111]. These peripheral B cell lymphomas routinely harbour amplified *Myc* genes that result from RAG-initiated dicentric translocations between the *IgH* J_H locus and sequences downstream of *Myc* [111] (see above). How RAG-initiated DSBs, which occur in progenitor B cells, could contribute to translocations and amplifications in mature B cells was an intriguing question. In this regard, prior studies suggested that RAG-generated breaks on chromosome 12 could be generated frequently due to the V(D)J joining defect associated with ATM deficiency and that the resulting telomere-deleted portions of chromosome 12 (*IgH* is near the telomere) could persist through development into mature B cells due to the G1 checkpoint defect associated with ATM deficiency [183]. Translocation cloning studies further revealed that such RAG-initiated DSBs in progenitor B cells are developmentally propagated into

mature B cells in the form of dicentric chromosomes. These dicentrics then undergo BFB cycles in ATM-deficient mature B cells to generate new DSBs in a large region of chromosome 12 downstream of the *IgH* locus that robustly translocate to DSBs near the *Myc* gene and undergo BFB amplification of *Myc* [49]. In the latter context, these ATM-deficient mouse mature B cell lymphomas share similar mechanisms of *Myc* amplification to mouse pro-B cell lymphomas deficient for both C-NHEJ and TP53 [13, 16].

3.8 Three-Dimensional Genome Organization and Translocations

Our current understanding of genome organization is derived from early cytogenetic studies [184] and more recent chromosome conformation capture (3C)-based methods [38, 185, 186]. In interphase nuclei, chromosomes are non-randomly organized and each chromosome fills a nuclear space or territory. At the 1–10 Mb scale, active and inactive regions exist in separate compartments and conform to a fractal globule capable of dynamic local compaction across the length of the chromosome [37]. Smaller topologically associated domains (TADs) of approximately 1 Mb ("1 Mb domains") exist within these compartments and comprise the majority of specific chromosomal contacts [185, 187, 188].

Translocations require DSBs at two independent sites and also require the two sites to be synapsed at the time they are broken. Various studies in yeast indicate increased chromatin mobility of sequences containing DSBs [42, 189]. Likewise, chromosomes with eroded telomeres, equivalent to DSBs, display 53BP1-dependent movement [43] and recent live cell tracking in mammalian systems which simultaneously follow interchromosomal I-*Sce*I DSBs from many cells displayed non-directional saltatory motion with increased pairing of interchromosomal DSBs over time [44]. Thus, increased movements of DSBs may contribute to their synapsis. In the absence of enforced movements, the frequency of a translocation can, in simplistic terms, be considered proportional to the frequency of un-joined DSBs at site 1 times the frequency of DSBs at site 2 times the frequency at which these DSBs are synapsed: $(DSB_{freq1}) \times (DSB_{freq2}) \times (Synapsis_{freq})$ [5]. These principles apply to spatially proximal and distal sites, and both in the context of developmentally programmed events such as V(D)J recombination and CSR or through spontaneous illicit joining to genome-wide DSBs.

Translocation cloning studies have clearly demonstrated that highly frequent DSBs can drive recurrent translocations irrespective of their relative average position in the genome [38, 39, 45, 46, 49, 118]. This phenomenon derives from the finding that spatial heterogeneity in 3D genome organization allows most genomic sites to be proximal in some cells in a population [38]. Thus, highly-frequent DSBs

can multiplicatively dominate the translocation frequency equation by greatly increasing the chance that two more rarely synapsed sites will be broken in cells in which they are synapsed [5, 38], allowing translocations across compartments that, on average, would be considered distal. In this regard, translocation cloning studies on G1-arrested ATM-deficient pro-B cell lines revealed that DSBs from eight independent I-*Sce*I DSB bait sites on various chromosomes translocated recurrently to five different antigen receptor loci on different chromosomes (40 pairs of loci); thus, recurrent detection of dominant antigen receptor locus DSBs translocated to dominant I-*Sce*I DSBs independent of chromosomal location due to 3D genome heterogeneity in the cell population (Fig. 3.2a) [38]. This explanation can also explain the dominance of AID hotspot DSBs in defining the translocation landscape of CSR activated B cells independent of chromosomal location (Fig. 3.3) [45, 46, 49].

In cases where particular DSBs are not dominant, synapsis frequency can play a much more dominant role in driving translocations. In experimental conditions, DSBs across the genome of ATM-deficient pro-B cell lines were normalized by treating cells with 5Gy of IR to induce, on average, 100 DSBs per cell. In such cases, antigen receptor locus DSBs were no longer so dominant and factors that increase synapsis frequency of two sequences became more influential (Fig. 3.2b) [38]. In accord with Hi-C mapping studies, such factors include placement in active versus inactive chromatin, associating with similarly-sized chromosomes, (more prominently) residing on the same chromosome *in cis* (demonstrated by SNP mapping), and (most prominently) lying within Mb domains *in cis* on a chromosome [5, 38]. A most striking feature of the greatly increased probability of sequences on the same chromosome lying proximal to each other was the finding that IR treatment of G1-arrested pro-B cells led the length of the *cis*-chromosome harbouring a bait DSB to become a major hotspot region for translocation of bait DSBs (Fig. 3.2b) [38].

Within a *cis*-chromosome, DSBs within Mb domains of a bait DSB have the highest frequency of translocation to the bait DSB [5, 38]. This phenomenon is thought to be due, at least in part, to sequences within such domains having a greater probability of being synapsed via Brownian (Langevin) motion [5, 38, 39]. In this regard, I-*Sce*I and/or Cas9:gRNA DSBs separated by 100 kb translocated to each other within the *IgH* locus and within the *Myc* locus in B cells, T cells or fibroblasts at frequencies high enough to support substantial *IgH* CSR [39]. These findings suggest CSR may have evolved to employ the high frequency synapsis of sequences in Mb domains, as opposed to or in addition to more specialized synapsis mechanisms, with the high frequency of AID-initiated DSBs to helping to drive physiological levels of CSR [5, 39]. Such mechanisms have also been implicated in synapsis of V, D, and J segments during V(D)J recombination [40] and may contribute to recurrent interstitial deletions found in T-ALLs and other cancers (e.g., [27, 190]).

References

1. Gainor JF, Shaw AT (2013) Novel targets in non-small cell lung cancer: ROS1 and RET fusions. Oncologist 18(7):865–875
2. Kuppers R (2005) Mechanisms of B-cell lymphoma pathogenesis. Nat Rev Cancer 5(4):251–262
3. Mitelman F, Johansson B, Mertens F (2007) The impact of translocations and gene fusions on cancer causation. Nat Rev Cancer 7(4):233–245
4. Nambiar M, Kari V, Raghavan SC (2008) Chromosomal translocations in cancer. Biochim Biophys Acta 1786(2):139–152
5. Alt FW et al (2013) Mechanisms of programmed DNA lesions and genomic instability in the immune system. Cell 152(3):417–429
6. Groffen J et al (1984) Philadelphia chromosomal breakpoints are clustered within a limited region, bcr, on chromosome 22. Cell 36(1):93–99
7. Lugo TG et al (1990) Tyrosine kinase activity and transformation potency of bcr-abl oncogene products. Science 247(4946):1079–1082
8. Nowell P, Hungerford D (1960) A minute chromosome in human chronic granulocytic leukemia [abstract]. Science 132:1497
9. Gostissa M, Alt FW, Chiarle R (2011) Mechanisms that promote and suppress chromosomal translocations in lymphocytes. Annu Rev Immunol 29:319–350
10. Robbiani DF, Nussenzweig MC (2013) Chromosome translocation, B cell lymphoma, and activation-induced cytidine deaminase. Annu Rev Pathol 8:79–103
11. Hnisz D et al (2013) Super-enhancers in the control of cell identity and disease. Cell 155(4):934–947
12. Alt FW et al (1978) Selective multiplication of dihydrofolate reductase genes in methotrexate-resistant variants of cultured murine cells. J Biol Chem 253(5):1357–1370
13. Difilippantonio MJ et al (2002) Evidence for replicative repair of DNA double-strand breaks leading to oncogenic translocation and gene amplification. J Exp Med 196(4):469–480
14. Papenhausen PR, Griffin S, Tepperberg J (2005) Oncogene amplification in transforming myelodysplasia. Exp Mol Pathol 79(2):168–175
15. Sharpless NE et al (2001) Impaired nonhomologous end-joining provokes soft tissue sarcomas harboring chromosomal translocations, amplifications, and deletions. Mol Cell 8(6):1187–1196
16. Zhu C et al (2002) Unrepaired DNA breaks in p53-deficient cells lead to oncogenic gene amplification subsequent to translocations. Cell 109(7):811–821
17. McClintock B (1941) The stability of broken ends of chromosomes in Zea mays. Genetics 26(2):234–282
18. Murnane JP (2012) Telomere dysfunction and chromosome instability. Mutat Res 730(1-2):28–36
19. Albertson DG et al (2003) Chromosome aberrations in solid tumors. Nat Genet 34(4):369–376
20. Rikova K et al (2007) Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. Cell 131(6):1190–1203
21. Albertson DG (2006) Gene amplification in cancer. Trends Genet 22(8):447–455
22. Munshi NC, Avet-Loiseau H (2011) Genomics in multiple myeloma. Clin Cancer Res 17(6):1234–1242
23. Zha S et al (2010) ATM-deficient thymic lymphoma is associated with aberrant tcrd rearrangement and gene amplification. J Exp Med 207(7):1369–1380
24. Burkhardt L et al (2013) CHD1 is a 5q21 tumor suppressor required for ERG rearrangement in prostate cancer. Cancer Res 73(9):2795–2805
25. Gutierrez A et al (2011) The BCL11B tumor suppressor is mutated across the major molecular subtypes of T-cell acute lymphoblastic leukemia. Blood 118(15):4169–4173

26. Krohn A et al (2014) Heterogeneity and chronology of PTEN deletion and ERG fusion in prostate cancer. Mod Pathol 27(12):1612–1620
27. Larmonie NS et al (2013) Breakpoint sites disclose the role of the V(D)J recombination machinery in the formation of T-cell receptor (TCR) and non-TCR associated aberrations in T-cell acute lymphoblastic leukemia. Haematologica 98(8):1173–1184
28. Gostissa M et al (2009) Chromosomal location targets different MYC family gene members for oncogenic translocations. Proc Natl Acad Sci U S A 106(7):2265–2270
29. Wang JH et al (2009) Mechanisms promoting translocations in editing and switching peripheral B cells. Nature 460(7252):231–236
30. Deriano L, Roth DB (2013) Modernizing the nonhomologous end-joining repertoire: alternative and classical NHEJ share the stage. Annu Rev Genet 47:433–455
31. Shiloh Y, Ziv Y (2013) The ATM protein kinase: regulating the cellular response to genotoxic stress, and more. Nat Rev Mol Cell Biol 14(4):197–210
32. Zhang Y et al (2010) The role of mechanistic factors in promoting chromosomal translocations found in lymphoid and other cancers. Adv Immunol 106:93–133
33. Symington LS, Gautier J (2011) Double-strand break end resection and repair pathway choice. Annu Rev Genet 45:247–271
34. Chapman JR, Taylor MR, Boulton SJ (2012) Playing the end game: DNA double-strand break repair pathway choice. Mol Cell 47(4):497–510
35. Boboila C, Alt FW, Schwer B (2012) Classical and alternative end-joining pathways for repair of lymphocyte-specific and general DNA double-strand breaks. Adv Immunol 116:1–49
36. Kim N, Jinks-Robertson S (2012) Transcription as a source of genome instability. Nat Rev Genet 13(3):204–214
37. Lieberman-Aiden E et al (2009) Comprehensive mapping of long-range interactions reveals folding principles of the human genome. Science 326(5950):289–293
38. Zhang Y et al (2012) Spatial organization of the mouse genome and its role in recurrent chromosomal translocations. Cell 148(5):908–921
39. Gostissa M et al (2014) IgH class switching exploits a general property of two DNA breaks to be joined in cis over long chromosomal distances. Proc Natl Acad Sci U S A 111(7):2644–2649
40. Lucas JS et al (2014) 3D trajectories adopted by coding and regulatory DNA elements: first-passage times for genomic interactions. Cell 158(2):339–352
41. Dion V, Gasser SM (2013) Chromatin movement in the maintenance of genome stability. Cell 152(6):1355–1364
42. Mine-Hattab J, Rothstein R (2012) Increased chromosome mobility facilitates homology search during recombination. Nat Cell Biol 14(5):510–517
43. Dimitrova N et al (2008) 53BP1 promotes non-homologous end joining of telomeres by increasing chromatin mobility. Nature 456(7221):524–528
44. Roukos V et al (2013) Spatial dynamics of chromosome translocations in living cells. Science 341(6146):660–664
45. Chiarle R et al (2011) Genome-wide translocation sequencing reveals mechanisms of chromosome breaks and rearrangements in B cells. Cell 147(1):107–119
46. Klein IA et al (2011) Translocation-capture sequencing reveals the extent and nature of chromosomal rearrangements in B lymphocytes. Cell 147(1):95–106
47. Haber JE (2006) Transpositions and translocations induced by site-specific double-strand breaks in budding yeast. DNA Repair (Amst) 5(9–10):998–1009
48. Zarrin AA et al (2007) Antibody class switching mediated by yeast endonuclease-generated DNA breaks. Science 315(5810):377–381
49. Hu J et al (2014) Developmental propagation of V(D)J recombination-associated DNA breaks and translocations in mature B cells via dicentric chromosomes. Proc Natl Acad Sci U S A 111(28):10269–10274
50. Kim YG, Cha J, Chandrasegaran S (1996) Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. Proc Natl Acad Sci U S A 93(3):1156–1160

51. Christian M et al (2010) Targeting DNA double-strand breaks with TAL effector nucleases. Genetics 186(2):757–761
52. Jinek M et al (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337(6096):816–821
53. Brunet E et al (2009) Chromosomal translocations induced at specified loci in human stem cells. Proc Natl Acad Sci U S A 106(26):10620–10625
54. Piganeau M et al (2013) Cancer translocations in human cells induced by zinc finger and TALE nucleases. Genome Res 23(7):1182–1193
55. Frock RL et al (2015) Genome-wide detection of DNA double-stranded breaks induced by engineered nucleases. Nat Biotechnol 33(2):179–86
56. Vilenchik MM, Knudson AG (2003) Endogenous DNA double-strand breaks: production, fidelity of repair, and induction of cancer. Proc Natl Acad Sci U S A 100(22):12871–12876
57. De Bont R, van Larebeke N (2004) Endogenous DNA damage in humans: a review of quantitative data. Mutagenesis 19(3):169–185
58. Aguilera A, Garcia-Muse T (2013) Causes of genome instability. Annu Rev Genet 47:1–32
59. Branzei D, Foiani M (2010) Maintaining genome stability at the replication fork. Nat Rev Mol Cell Biol 11(3):208–219
60. Hu J et al (2012) The intra-S phase checkpoint targets Dna2 to prevent stalled replication forks from reversing. Cell 149(6):1221–1232
61. Lambert S, Carr AM (2013) Impediments to replication fork movement: stabilisation, reactivation and genome instability. Chromosoma 122(1–2):33–45
62. Ferber MJ et al (2004) Positioning of cervical carcinoma and Burkitt lymphoma translocation breakpoints with respect to the human papillomavirus integration cluster in FRA8C at 8q24.13. Cancer Genet Cytogenet 154(1):1–9
63. Morelli C et al (2002) Cloning and characterization of the common fragile site FRA6F harboring a replicative senescence gene and frequently deleted in human tumors. Oncogene 21(47):7266–7276
64. Sinclair PB et al (2004) A fluorescence in situ hybridization map of 6q deletions in acute lymphocytic leukemia: identification and analysis of a candidate tumor suppressor gene. Cancer Res 64(12):4089–4098
65. Barlow JH et al (2013) Identification of early replicating fragile sites that contribute to genome instability. Cell 152(3):620–632
66. Fong YW, Cattoglio C, Tjian R (2013) The intertwined roles of transcription and repair proteins. Mol Cell 52(3):291–302
67. Helmrich A, Ballarino M, Tora L (2011) Collisions between replication and transcription complexes cause common fragile site instability at the longest human genes. Mol Cell 44(6):966–977
68. Prado F, Aguilera A (2005) Impairment of replication fork progression mediates RNA polII transcription-associated recombination. EMBO J 24(6):1267–1276
69. Haffner MC et al (2011) Transcription-induced DNA double strand breaks: both oncogenic force and potential therapeutic target? Clin Cancer Res 17(12):3858–3864
70. Li X, Manley JL (2005) Inactivation of the SR protein splicing factor ASF/SF2 results in genomic instability. Cell 122(3):365–378
71. Shinkura R et al (2003) The influence of transcriptional orientation on endogenous switch region function. Nat Immunol 4(5):435–441
72. Yu K et al (2003) R-loops at immunoglobulin class switch regions in the chromosomes of stimulated B cells. Nat Immunol 4(5):442–451
73. Curtin NJ (2012) DNA repair dysregulation from cancer driver to therapeutic target. Nat Rev Cancer 12(12):801–817
74. Mehta A, Haber JE (2014) Sources of DNA double-strand breaks and models of recombinational DNA repair. Cold Spring Harb Perspect Biol 6(9):a016428
75. Asaithamby A, Chen DJ (2009) Cellular responses to DNA double-strand breaks after low-dose gamma-irradiation. Nucleic Acids Res 37(12):3912–3923

76. Joannides M, Grimwade D (2010) Molecular biology of therapy-related leukaemias. Clin Transl Oncol 12(1):8–14
77. Joannides M et al (2011) Molecular pathogenesis of secondary acute promyelocytic leukemia. Mediterr J Hematol Infect Dis 3(1), e2011045
78. Helmink BA, Sleckman BP (2012) The response to and repair of RAG-mediated DNA double-strand breaks. Annu Rev Immunol 30:175–202
79. Janz S (2006) Myc translocations in B cell and plasma cell neoplasms. DNA Repair (Amst) 5(9–10):1213–1224
80. Krangel MS (2009) Mechanics of T cell receptor gene rearrangement. Curr Opin Immunol 21(2):133–139
81. Nishana M, Raghavan SC (2012) Role of recombination activating genes in the generation of antigen receptor diversity and beyond. Immunology 137(4):271–281
82. Schatz DG, Baltimore D (2004) Uncovering the V(D)J recombinase. Cell 116(2 Suppl):S103–S106, 2 p following S106
83. Fugmann SD et al (2000) The RAG proteins and V(D)J recombination: complexes, ends, and transposition. Annu Rev Immunol 18:495–527
84. Schatz DG, Swanson PC (2011) V(D)J recombination: mechanisms of initiation. Annu Rev Genet 45:167–202
85. Bossen C, Mansson R, Murre C (2012) Chromatin topology and the regulation of antigen receptor assembly. Annu Rev Immunol 30:337–356
86. Hewitt SL, Chaumeil J, Skok JA (2010) Chromosome dynamics and the regulation of V(D)J recombination. Immunol Rev 237(1):43–54
87. Desiderio S (2010) Temporal and spatial regulatory functions of the V(D)J recombinase. Semin Immunol 22(6):362–369
88. Ji Y et al (2010) The in vivo pattern of binding of RAG1 and RAG2 to antigen receptor loci. Cell 141(3):419–431
89. Matthews AG, Oettinger MA (2009) RAG: a recombinase diversified. Nat Immunol 10(8):817–821
90. Schatz DG, Ji Y (2011) Recombination centres and the orchestration of V(D)J recombination. Nat Rev Immunol 11(4):251–263
91. Taccioli GE et al (1993) Impairment of V(D)J recombination in double-strand break repair mutants. Science 260(5105):207–210
92. Alt FW, Baltimore D (1982) Joining of immunoglobulin heavy chain gene segments: implications from a chromosome with evidence of three D-JH fusions. Proc Natl Acad Sci U S A 79(13):4118–4122
93. Chaudhuri J et al (2007) Evolution of the immunoglobulin heavy chain class switch recombination mechanism. Adv Immunol 94:157–214
94. Honjo T, Kinoshita K, Muramatsu M (2002) Molecular mechanism of class switch recombination: linkage with somatic hypermutation. Annu Rev Immunol 20:165–196
95. Jung D et al (2006) Mechanism and control of V(D)J recombination at the immunoglobulin heavy chain locus. Annu Rev Immunol 24:541–570
96. Muramatsu M et al (2000) Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. Cell 102(5):553–563
97. Peled JU et al (2008) The biochemistry of somatic hypermutation. Annu Rev Immunol 26:481–511
98. Rogozin IB, Diaz M (2004) Cutting edge: DGYW/WRCH is a better predictor of mutability at G:C bases in Ig hypermutation than the widely accepted RGYW/WRCY motif and probably reflects a two-step activation-induced cytidine deaminase-triggered process. J Immunol 172(6):3382–3384
99. Hackney JA et al (2009) DNA targets of AID evolutionary link between antibody somatic hypermutation and class switch recombination. Adv Immunol 101:163–189
100. Storb U (2014) Why does somatic hypermutation by AID require transcription of its target genes? Adv Immunol 122:253–277

101. Di Noia JM, Neuberger MS (2007) Molecular mechanisms of antibody somatic hypermutation. Annu Rev Biochem 76:1–22
102. Stavnezer J, Guikema JE, Schrader CE (2008) Mechanism and regulation of class switch recombination. Annu Rev Immunol 26:261–292
103. Daniel JA, Nussenzweig A (2013) The AID-induced DNA damage response in chromatin. Mol Cell 50(3):309–321
104. Keim C et al (2013) Regulation of AID, the B-cell genome mutator. Genes Dev 27(1):1–17
105. Matthews AJ et al (2014) Regulation of immunoglobulin class-switch recombination: choreography of noncoding transcription, targeted DNA deamination, and long-range DNA repair. Adv Immunol 122:1–57
106. Wuerffel R et al (2007) S-S synapsis during class switch recombination is promoted by distantly located transcriptional elements and activation-induced deaminase. Immunity 27(5):711–722
107. Bednarski JJ, Sleckman BP (2012) Lymphocyte development: integration of DNA damage response signaling. Adv Immunol 116:175–204
108. Chaumeil J et al (2013) Higher-order looping and nuclear organization of Tcra facilitate targeted rag cleavage and regulated rearrangement in recombination centers. Cell Rep 3(2):359–370
109. Deriano L et al (2011) The RAG2 C terminus suppresses genomic instability and lymphomagenesis. Nature 471(7336):119–123
110. Dyer MJ et al (2010) Immunoglobulin heavy chain locus chromosomal translocations in B-cell precursor acute lymphoblastic leukemia: rare clinical curios or potent genetic drivers? Blood 115(8):1490–1499
111. Tepsuporn S et al (2014) Mechanisms that can promote peripheral B-cell lymphoma in ATM-deficient mice. Cancer Immunol Res 2(9):857–866
112. Wesemann DR et al (2013) Microbial colonization influences early B-lineage development in the gut lamina propria. Nature 501(7465):112–115
113. Merelli I et al (2010) RSSsite: a reference database and prediction tool for the identification of cryptic recombination signal sequences in human and murine genomes. Nucleic Acids Res 38(Web Server issue):W262–W267
114. Tsai AG, Lieber MR (2010) Mechanisms of chromosomal rearrangement in the human genome. BMC Genomics 11(Suppl 1):S1
115. Mendes RD et al (2014) PTEN microdeletions in T-cell acute lymphoblastic leukemia are caused by illegitimate RAG-mediated recombination events. Blood 124(4):567–578
116. Pasqualucci L et al (2001) Hypermutation of multiple proto-oncogenes in B-cell diffuse large-cell lymphomas. Nature 412(6844):341–346
117. Liu M et al (2008) Two levels of protection for the B cell genome during somatic hypermutation. Nature 451(7180):841–845
118. Hakim O et al (2012) DNA damage defines sites of recurrent chromosomal translocations in B lymphocytes. Nature 484(7392):69–74
119. Pavri R, Nussenzweig MC (2011) AID targeting in antibody diversity. Adv Immunol 110:1–26
120. Liu M, Schatz DG (2009) Balancing AID and DNA repair during somatic hypermutation. Trends Immunol 30(4):173–181
121. Pasqualucci L et al (2008) AID is required for germinal center-derived lymphomagenesis. Nat Genet 40(1):108–112
122. Meng F-L et al (2014) Convergent transcription at intragenic super-enhancers targets AID-initiated genomic instability. Cell 159(7):1538–1548
123. Lieber MR (2010) The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. Annu Rev Biochem 79:181–211
124. Sun J et al (2012) Human Ku70/80 protein blocks exonuclease 1-mediated DNA resection in the presence of human Mre11 or Mre11/Rad50 protein complex. J Biol Chem 287(7):4936–4945

125. Kumar V, Alt FW, Oksenych V (2014) Functional overlaps between XLF and the ATM-dependent DNA double strand break response. DNA Repair (Amst) 16:11–22
126. Buck D et al (2006) Cernunnos, a novel nonhomologous end-joining factor, is mutated in human immunodeficiency with microcephaly. Cell 124(2):287–299
127. Du L et al (2012) Cernunnos influences human immunoglobulin class switch recombination and may be associated with B cell lymphomagenesis. J Exp Med 209(2):291–305
128. Ahnesorg P, Smith P, Jackson SP (2006) XLF interacts with the XRCC4-DNA ligase IV complex to promote DNA nonhomologous end-joining. Cell 124(2):301–313
129. Lu H et al (2007) Length-dependent binding of human XLF to DNA and stimulation of XRCC4.DNA ligase IV activity. J Biol Chem 282(15):11155–11162
130. Mahaney BL et al (2013) XRCC4 and XLF form long helical protein filaments suitable for DNA end protection and alignment to facilitate DNA double strand break repair. Biochem Cell Biol 91(1):31–41
131. Tsai CJ, Kim SA, Chu G (2007) Cernunnos/XLF promotes the ligation of mismatched and noncohesive DNA ends. Proc Natl Acad Sci U S A 104(19):7851–7856
132. de Villartay JP (2009) V(D)J recombination deficiencies. Adv Exp Med Biol 650:46–58
133. van der Burg M et al (2009) A DNA-PKcs mutation in a radiosensitive T-B- SCID patient inhibits Artemis activation and nonhomologous end-joining. J Clin Invest 119(1):91–98
134. Frank KM et al (1998) Late embryonic lethality and impaired V(D)J recombination in mice lacking DNA ligase IV. Nature 396(6707):173–177
135. Gao Y et al (1998) A critical role for DNA end-joining proteins in both lymphogenesis and neurogenesis. Cell 95(7):891–902
136. Gu Y et al (2000) Defective embryonic neurogenesis in Ku-deficient but not DNA-dependent protein kinase catalytic subunit-deficient mice. Proc Natl Acad Sci U S A 97(6):2668–2673
137. Frank KM et al (2000) DNA ligase IV deficiency in mice leads to defective neurogenesis and embryonic lethality via the p53 pathway. Mol Cell 5(6):993–1002
138. Gao Y et al (2000) Interplay of p53 and DNA-repair protein XRCC4 in tumorigenesis, genomic stability and development. Nature 404(6780):897–900
139. Rooney S, Chaudhuri J, Alt FW (2004) The role of the non-homologous end-joining pathway in lymphocyte development. Immunol Rev 200(1):115–131
140. Frappart PO et al (2009) Recurrent genomic alterations characterize medulloblastoma arising from DNA double-strand break repair deficiency. Proc Natl Acad Sci U S A 106(6):1880–1885
141. Yan CT et al (2006) XRCC4 suppresses medulloblastomas with recurrent translocations in p53-deficient mice. Proc Natl Acad Sci U S A 103(19):7378–7383
142. Li G et al (2008) Lymphocyte-specific compensation for XLF/cernunnos end-joining functions in V(D)J recombination. Mol Cell 31(5):631–640
143. Boboila C et al (2010) Alternative end-joining catalyzes robust IgH locus deletions and translocations in the combined absence of ligase 4 and Ku70. Proc Natl Acad Sci U S A 107(7):3034–3039
144. Ferguson DO et al (2000) The interplay between nonhomologous end-joining and cell cycle checkpoint factors in development, genomic stability, and tumorigenesis. Cold Spring Harb Symp Quant Biol 65:395–403
145. Wang JH et al (2008) Oncogenic transformation in the absence of Xrcc4 targets peripheral B cells that have undergone editing and switching. J Exp Med 205(13):3079–3090
146. Boulton SJ, Jackson SP (1996) Saccharomyces cerevisiae Ku70 potentiates illegitimate DNA double-strand break repair and serves as a barrier to error-prone DNA repair pathways. EMBO J 15(18):5093–5103
147. Kabotyanski EB et al (1998) Double-strand break repair in Ku86- and XRCC4-deficient cells. Nucleic Acids Res 26(23):5333–5342
148. Boboila C et al (2010) Alternative end-joining catalyzes class switch recombination in the absence of both Ku70 and DNA ligase 4. J Exp Med 207(2):417–427
149. Yan CT et al (2007) IgH class switching and translocations use a robust non-classical end-joining pathway. Nature 449(7161):478–482

150. Stephens PJ et al (2009) Complex landscapes of somatic rearrangement in human breast cancer genomes. Nature 462(7276):1005–1010
151. Zhang Y, Rowley JD (2006) Chromatin structural elements and chromosomal translocations in leukemia. DNA Repair (Amst) 5(9–10):1282–1297
152. Grabarz A et al (2012) Initiation of DNA double strand break repair: signaling and single-stranded resection dictate the choice between homologous recombination, non-homologous end-joining and alternative end-joining. Am J Cancer Res 2(3):249–268
153. Guirouilh-Barbat J et al (2007) Defects in XRCC4 and KU80 differentially affect the joining of distal nonhomologous ends. Proc Natl Acad Sci U S A 104(52):20902–20907
154. Komori T, Sugiyama H (1993) N sequences, P nucleotides and short sequence homologies at junctional sites in VH to VHDJH and VHDJH to JH joining. Mol Immunol 30(16): 1393–1398
155. Stavnezer J et al (2010) Mapping of switch recombination junctions, a tool for studying DNA repair pathways during immunoglobulin class switching. Adv Immunol 108:45–109
156. Simsek D, Jasin M (2010) Alternative end-joining is suppressed by the canonical NHEJ component Xrcc4-ligase IV during chromosomal translocation formation. Nat Struct Mol Biol 17(4):410–416
157. Ghezraoui H et al (2014) Chromosomal translocations in human cells are generated by canonical nonhomologous end-joining. Mol Cell 55(6):829–842
158. Perlman S, Becker-Catania S, Gatti RA (2003) Ataxia-telangiectasia: diagnosis and treatment. Semin Pediatr Neurol 10(3):173–182
159. Choi M et al (2012) Attractor landscape analysis reveals feedback loops in the p53 network that control the cellular response to DNA damage. Sci Signal 5(251):ra83
160. Nussenzweig A, Nussenzweig MC (2010) Origin of chromosomal translocations in lymphoid cancer. Cell 141(1):27–38
161. Scully R, Xie A (2013) Double strand break repair functions of histone H2AX. Mutat Res 750(1–2):5–14
162. Al-Hakim A et al (2010) The ubiquitous role of ubiquitin in the DNA damage response. DNA Repair (Amst) 9(12):1229–1240
163. Panier S, Boulton SJ (2014) Double-strand break repair: 53BP1 comes into focus. Nat Rev Mol Cell Biol 15(1):7–18
164. Sirbu BM, Cortez D (2013) DNA damage response: three levels of DNA repair regulation. Cold Spring Harb Perspect Biol 5(8):a012724
165. Zimmermann M, de Lange T (2014) 53BP1: pro choice in DNA repair. Trends Cell Biol 24(2):108–117
166. Bassing CH, Alt FW (2004) The cellular response to general and programmed DNA double strand breaks. DNA Repair (Amst) 3(8–9):781–796
167. Franco S, Alt FW, Manis JP (2006) Pathways that suppress programmed DNA breaks from progressing to chromosomal breaks and translocations. DNA Repair (Amst) 5(9-10): 1030–1041
168. Lavin MF (2008) Ataxia-telangiectasia: from a rare disorder to a paradigm for cell signalling and cancer. Nat Rev Mol Cell Biol 9(10):759–769
169. Xu Y et al (1996) Targeted disruption of ATM leads to growth retardation, chromosomal fragmentation during meiosis, immune defects, and thymic lymphoma. Genes Dev 10(19):2411–2422
170. Bassing CH et al (2002) Increased ionizing radiation sensitivity and genomic instability in the absence of histone H2AX. Proc Natl Acad Sci U S A 99(12):8173–8178
171. Franco S et al (2006) H2AX prevents DNA breaks from progressing to chromosome breaks and translocations. Mol Cell 21(2):201–214
172. Bassing CH et al (2003) Histone H2AX: a dosage-dependent suppressor of oncogenic translocations and tumors. Cell 114(3):359–370
173. Celeste A et al (2003) H2AX haploinsufficiency modifies genomic stability and tumor susceptibility. Cell 114(3):371–383

174. Ramiro AR et al (2006) Role of genomic instability and p53 in AID-induced c-myc-Igh translocations. Nature 440(7080):105–109
175. Manis JP et al (2004) 53BP1 links DNA damage-response pathways to immunoglobulin heavy chain class-switch recombination. Nat Immunol 5(5):481–487
176. Ward IM et al (2004) 53BP1 is required for class switch recombination. J Cell Biol 165(4):459–464
177. Celeste A et al (2002) Genomic instability in mice lacking histone H2AX. Science 296(5569):922–927
178. Zha S et al (2011) ATM damage response and XLF repair factor are functionally redundant in joining DNA breaks. Nature 469(7329):250–254
179. Liu X et al (2012) Overlapping functions between XLF repair protein and 53BP1 DNA damage response factor in end joining and lymphocyte development. Proc Natl Acad Sci U S A 109(10):3903–3908
180. Oksenych V et al (2012) Functional redundancy between repair factor XLF and damage response mediator 53BP1 in V(D)J recombination and DNA repair. Proc Natl Acad Sci U S A 109(7):2455–2460
181. Gumy-Pause F, Wacker P, Sappino AP (2004) ATM gene and lymphoid malignancies. Leukemia 18(2):238–242
182. McKinnon PJ (2012) ATM and the molecular pathogenesis of ataxia telangiectasia. Annu Rev Pathol 7:303–321
183. Callen E et al (2007) ATM prevents the persistence and propagation of chromosome breaks in lymphocytes. Cell 130(1):63–75
184. Roix JJ et al (2003) Spatial proximity of translocation-prone gene loci in human lymphomas. Nat Genet 34(3):287–291
185. Dixon JR et al (2012) Topological domains in mammalian genomes identified by analysis of chromatin interactions. Nature 485(7398):376–380
186. Nagano T et al (2013) Single-cell Hi-C reveals cell-to-cell variability in chromosome structure. Nature 502(7469):59–64
187. Dekker J, Marti-Renom MA, Mirny LA (2013) Exploring the three-dimensional organization of genomes: interpreting chromatin interaction data. Nat Rev Genet 14(6):390–403
188. Gibcus JH, Dekker J (2013) The hierarchy of the 3D genome. Mol Cell 49(5):773–782
189. Dion V et al (2012) Increased mobility of double-strand breaks requires Mec1, Rad9 and the homologous recombination machinery. Nat Cell Biol 14(5):502–509
190. Onozawa M, Aplan PD (2012) Illegitimate V(D)J recombination involving nonantigen receptor loci in lymphoid malignancy. Genes Chromosom Cancer 51(6):525–535

Chapter 4
Chromosome Translocations, Cancer Initiation and Clonal Evolution

Mel Greaves and Anthony M. Ford

Contents

4.1 Introduction 54
4.2 Common Translocation in Acute Lymphoblastic Leukaemia 54
4.3 Generation of the t(12;21) and *ETV6-RUNX1* Fusion 55
4.4 *In Utero* Origins of t(12;21) and Other Common Translocations 56
4.5 *ETV6-RUNX1* Fusion as an Initiating Event or Founder Mutation in ALL 60
4.6 Transforming and Maintenance Functions of ETV6-RUNX1 Protein 61
4.7 Secondary Mutations that Complement *ETV6-RUNX1* Fusion 64
4.8 The Contextual Role of *ETV6-RUNX1* Fusion in the Clonal Evolution of ALL 65
4.9 Other Translocations as Initiating Lesions in Leukaemia and Cancer 66
References 67

Abstract Chromosome translocations and the resultant fusion genes are highly recurrent and selectively associated with particular subtypes of blood cell cancers, sarcomas and some other common adult tumours including prostate cancer. Malignant progression is, however, driven as an evolutionary process by the accumulation of multiple genetic alterations that collectively impact on cell fitness. The functional impact and timing of individual changes, such as a fusion gene, may be dependent upon context of cell type, other concurrent genetic abnormalities and selective pressures in the micro-environment. In this chapter, we use *ETV6-RUNX1* in childhood acute lymphoblastic leukaemia (ALL) as a well-worked example of a chimaeric fusion gene which is likely an initiating or founder event in cancer clone evolution.

Keywords Clonal evolution • Fusion gene • Founder lesion • In utero • Twins

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4.1 Introduction

All cancers evolve by a Darwinian process of genetic diversification and sub-clonal selection within tissue ecosystems [1]. Mutations in cancer, as in evolution in general, arise stochastically or randomly with respect to the functions they encode, but a subset of maybe a few hundred in total are recurrent [2]. These 'driver' mutations are not autonomous acting 'selfish' genes but are positively selected on the basis of the fitness they contribute to the cell's phenotype in relation to the prevailing selection pressures. This is most clearly evident with drug resistance mutations but is true in general.

With the advent of multi-cellularity some 600 Myr ago, there was an intrinsic risk of single cell escapees, particularly in the totipotent or stem compartments of more complex, longer lived animals [3]. Multiple restraints including the early evolutionary innovation of 'suppressor' genes were therefore imposed [4]. It follows that for a fully-fledged malignancy to emerge, it needs to accumulate a set of mutations that collectively empower the cell and its descendent progeny to breach the multiple restraints, be they in negative feedback signalling or architectural. Every conceivable type of DNA mutation that occurs in nature is found in cancer cells from single nucleotide changes to gene copy number alterations and the more dramatic or gymnastic chromosomal translocations. The result, if it is to have selective currency in tumour evolution, is a change in the quality (or specificity) or quantity of a protein product. Chromosome translocations can do both.

Inter-chromosomal translocations were the first consistent or recurrent structural, genetic abnormalities discovered in human cancer [5] and the subsequent deciphering of the direct functional consequences of these chimaeric recombinants, and their modelling in mice, provided compelling evidence that they play a pivotal role in cellular transformation and tumour evolution [6, 7]. The protein products of translocations vary widely in their generic functions including transcriptional, cell cycle or apoptosis regulators suggesting that they contribute to many of the hallmark phenotypic features of cancer [8]. What is less clear is the timing of chromosome translocations and their place in the sequence of genetic events that initiates and drives clonal evolution. In this chapter, we use the t(12;21) translocation and its *ETV6-RUNX1* fusion product in leukaemia as a well-worked example whose status as an early and likely founder event in cancer clonal evolution is established.

4.2 Common Translocation in Acute Lymphoblastic Leukaemia

Multiple chromosomal translocations occur in subtypes of childhood and adult ALL [9, 10] (see also chapter by T Look) but three are common. The chromosome translocation t(12;21)(p13.2;q22.3) is the most frequent translocation in childhood leukaemia and paediatric cancer overall, but for many years was missed by

cytogeneticists because of the cryptic nature of the q22.3 alteration [11]. By molecular cloning, the t(12;21) was found to generate an in frame *ETV6-RUNX1* (also known as *TEL-AML1*) chimaeric fusion [12, 13]. The translocation is restricted to B cell precursor ($CD19^+$ $CD10^+$) ALL with a prevalence of ~25 %. Cases with *ETV6-RUNX1* have a typical incidence peak (as with hyperdiploid cases) at 3–5 years and are rare in adult ALL [14]. In early clinical studies, ALL cases with *ETV6-RUNX1* had an overall good prognosis but a significant fraction of cases were observed to relapse late (>2 years from diagnosis) [15]. More recent clinical studies with modified chemotherapeutic doses and schedules report a long term remission rate and possible cure approaching 100 % for this common sub-type [16].

Chimaeric fusions of the *KMT2A*/*MLL1* gene at 11q23.3 with various partners but predominantly *AFF4*/*AF4* at 4q21.3 have a high frequency (~75 %) in infant pro-B ($CD19^+$ $CD10^-$) ALL in infants (0–18 months) [10]. These cases are considered high risk but there have been recent improvements in their clinical outcome. The t(9;22)(q34.1;q11.2) *BCR-ABL1* translocation is present in around 5 % of childhood B cell precursor ALL and in a larger fraction of adult ALL [10]. These cases have a poor prognosis but this has also improved with more aggressive treatments, the introduction of imatinib and other ABL1 kinase inhibitors and bone marrow transplantation [17].

There is evidence that all three of these translocations are probably initiating or founder genetic events in ALL and for most cases of paediatric ALL, the *ETV6-RUNX1* , *KMT2A-AFF4* and at least some *BCR-ABL1* originate pre-natally *in utero*.

4.3 Generation of the t(12;21) and *ETV6-RUNX1* Fusion

As with other chromosome translocations [18, 19], t(12;21) is generated by double-stranded DNA breaks (DSB) followed by 'illegitimate' recombination. The mechanism of DSB formation is unknown and does not, in contrast to many other B cell lineage translocations, involve CpG islands or heptamer-nonamer sequences implicating off-target effects of the AICDA/AID and RAG IGH recombination machinery [18, 19]. Amplification of *ETV6-RUNX1* genomic fusions by long range inverse PCR, sequencing and mapping of breakpoints revealed that breaks are clustered within the 10 kb of the intron of *ETV6* between exons 5 and 6 and over the ~100 kb of the first intron of *RUNX1*, but are randomly distributed within these clustered regions [20] (Fig. 4.1).

The random or idiosyncratic nature of *ETV6-RUNX1* breakpoints (within the cluster region) results in each patient having a unique *ETV6-RUNX1* genomic fusion sequence. This provides a stable, specific and sensitive marker for clonal tracking both for minimal residual disease following or during drug treatment and for back-tracking the pre-clinical origins of disease. Micro-homologies at the breakpoint fusion regions suggest that non-homologous end-joining is the probable mechanism of recombination [20]. The reciprocal *ETV6-RUNX1* fusion is present in most cases

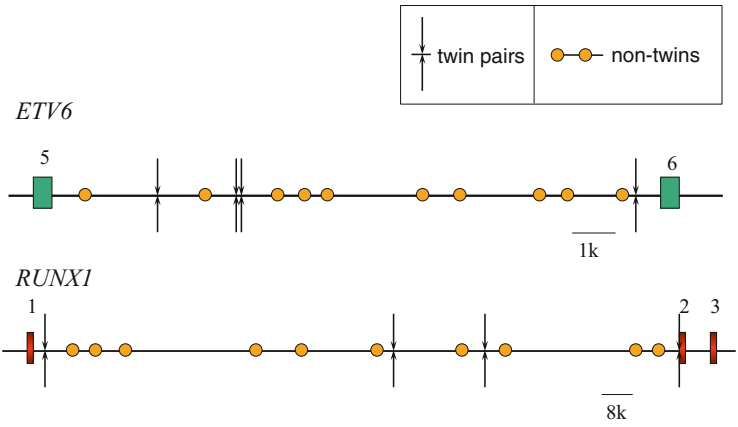

Fig. 4.1 Clonotypic genomic breakpoints in ETV6 and RUNX1 in singletons and monozygotic twins with ALL (Data reviewed in [21, 22])

and it has been assumed that the translocation is balanced or reciprocal. But recent genomic sequencing studies has found that at least in a small fraction of cases (~5 %) may have generated *ETV6-RUNX1* fusion as a consequence of a complex cascade of chromosome breaks and rearrangements [23, 24] (Fig. 4.2) similar to that described for the *ERG* fusions in prostate cancer [25].

4.4 *In Utero* Origins of t(12;21) and Other Common Translocations

Multiple lines of evidence have established that in most cases of childhood ALL with *ETV6-RUNX1*, the translocation arises pre-natally, *in utero* [21] (Fig. 4.3).

Identical twins with concordant ALL with t(12;21) share the same *ETV6-RUNX1* genomic fusion sequence (Fig. 4.1) [22, 26]. The latter is non-constitutive and its sharing in monozygotic, monochorionic twins reflects an *in utero* origin in one twin followed by dissemination of clonal progeny to the second twin via intra-placental anastomoses [22] (Fig. 4.4).

The comparative genomics of twins with concordant ALL reveals that whilst *ETV6-RUNX1* is shared or identical, all other recurrent and presumed 'driver' genetic changes including copy number alterations (CNA) and single nucleotide variants (SNV) are distinctive [23, 27] (Fig. 4.4).

These data suggest *ETV6-RUNX1* fusion may be sufficient by itself to initiate ALL and that CNA and SNV contribute to the molecular pathogenesis and clonal evolution in ALL as necessary but secondary, post-natal events.

Similarly, infant monozygotic twins that are concordant for *KMT2A*/*MLL*$^{+}$ ALL share identical fusion genomic rearrangements [22, 30]. One pair of twins with

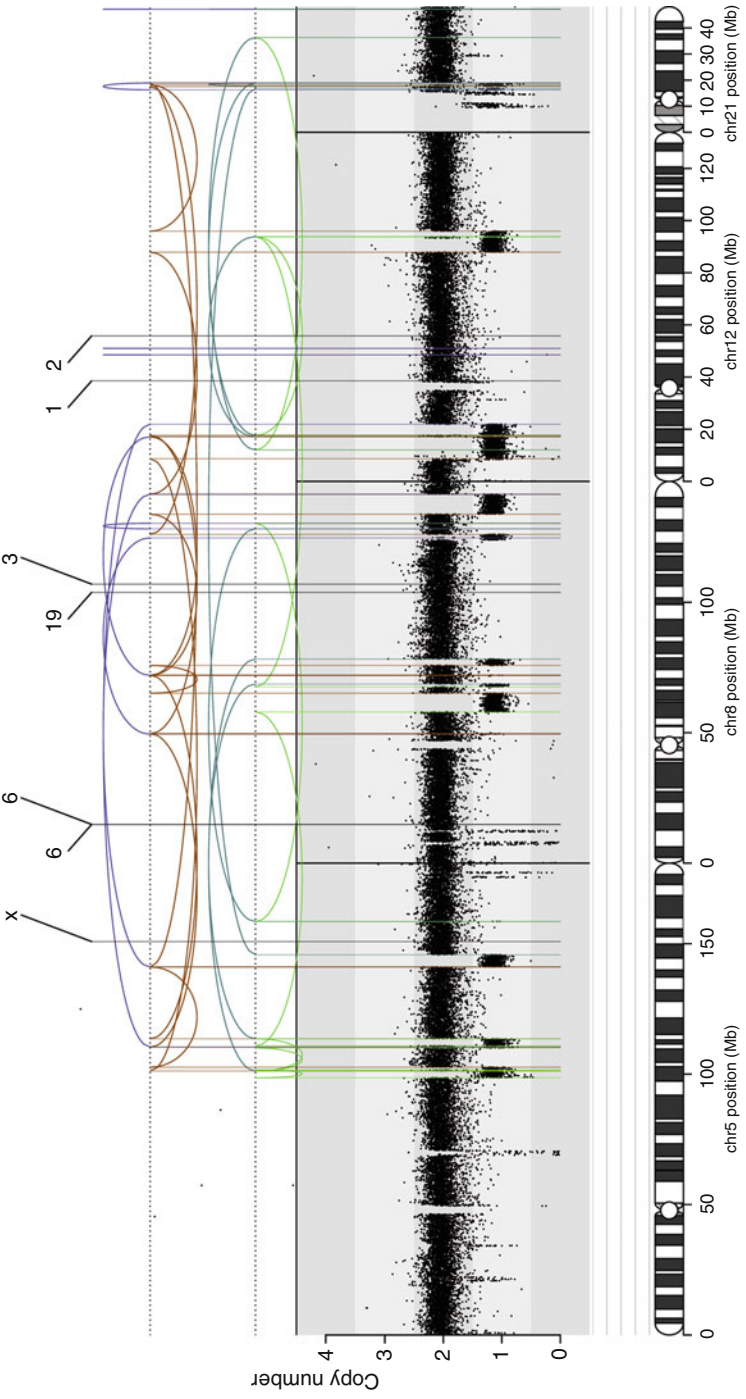

Fig. 4.2 Cascading chromosomal breaks generating an *ETV6-RUNX1* fusion based on paired end-sequencing of diagnostic DNA from a case of childhood ALL [24]. *Top, coloured lines* – chromosomal segment exchanges. *Bottom panel*, DNA copy number deletions (Figure courtesy of Yilong Li (The Wellcome Trust Sanger Institute))

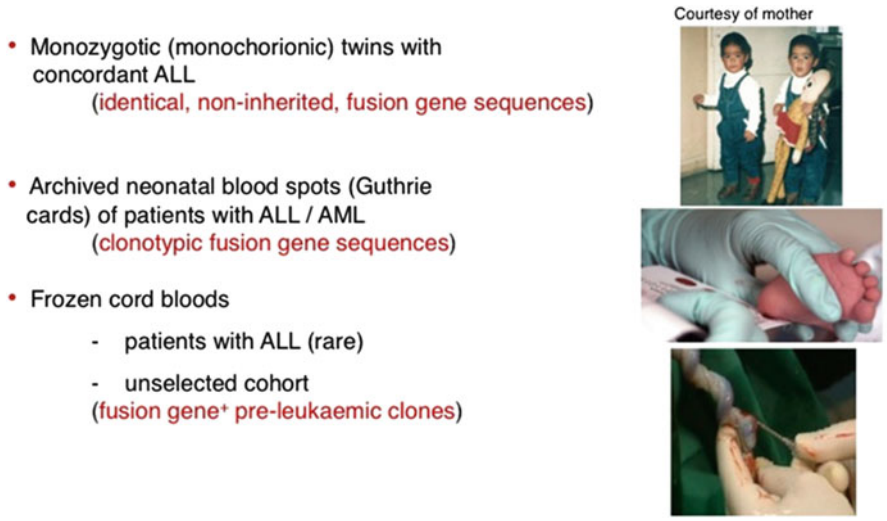

Fig. 4.3 Backtracking the pre-natal origins of childhood ALL (Data reviewed in ref. [21, 22])

concordant *BCR-ABL1*$^+$ ALL shared the same genomic fusion sequence but with distinctive secondary genetic changes [31]. Interestingly the twin where *BCR-ABL1* fusion was complemented by hyperdiploidy has survived ~8 years and remained in remission whilst the co-twin with the same *BCR-ABL1* fusion but an *IKZF1* deletion died in relapse after ~18 months (after receiving the same treatment) [31]. This observation suggests that secondary genetic changes in ALL can have a major impact on malignancy of disease and treatment response.

The concordance rate of ALL in monozygotic twins is close to 100 % in infant ALL but ~15 % in older children [22]. The very high rate in infants suggests that the *KMT2A*/*MLL* fusion may be sufficient for (rapid) leukaemogenesis and this is endorsed by recent genomic sequencing [32]. For childhood ALL with *ETV6-RUNX1*$^+$ ALL, the lower concordance rate is compatible with the notion that additional genetic changes are required for overt, clinical leukaemia. This is endorsed by the finding that in cases of *dis*cordant twins, the healthy co-twin carries a putative pre-leukaemic clone with the common *ETV6-RUNX1* fusion [27, 28] or *BCR-ABL1* fusion [31] but without the secondary changes present in the co-twin with ALL (Fig. 4.4).

Direct evidence for a pre-natal origin of t(12;21) in non-twinned or singleton patients comes from the detection of patient-specific *ETV6-RUNX1* [33] or *KMT2A-AFF4* [34] genomic sequences in archived neonatal blood spots (or Guthrie cards) of patients (Fig. 4.3). Similar neonatal blood spot evidence has been provided for *RUNX1-RUNX1T1* (*AML1-ETO*) fusions in childhood acute myeloid leukaemia (AML) [35]. Not all blood spots register positive however [21]. This could either mean that in some cases the common fusion genes in childhood ALL and AML arise post-natally or that the pre-leukaemic clone is present at a very low, undetectable frequency in the blood at birth (<1 in 30,000 cells). Screening of a large cohort of

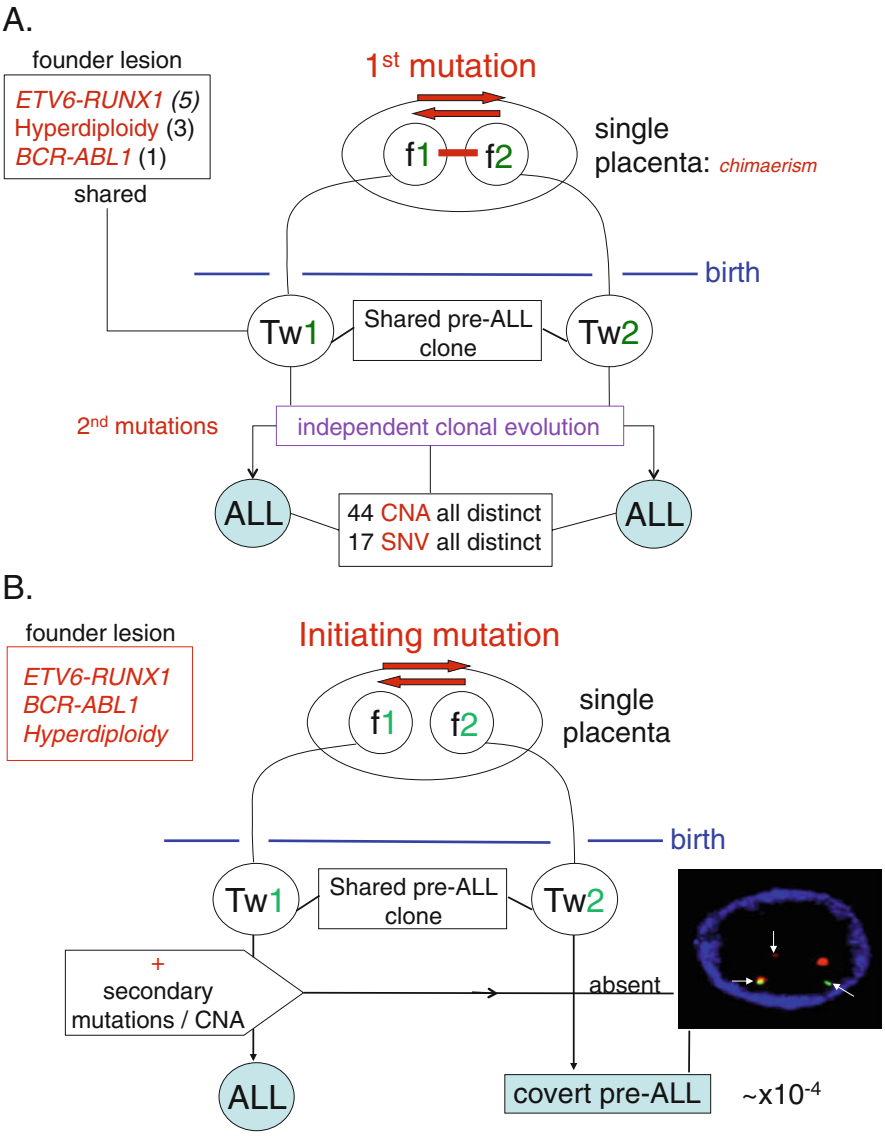

Fig. 4.4 Genomics of ALL in monozygotic twins. (**a**) 9 pairs of twins *con*cordant for ALL (Data in refs. [22, 23, 27]). (**b**) 3 pairs of twins *dis*cordant for ALL (Data in refs. [22, 28, 29, 31]). FISH image on *right* shows putative pre-leukaemic cell with yellow fusion (*ETV6-RUNX1*) signal. The normal *ETV6* allele (*green*, *arrowed*) is retained. Small remants of rearranged *RUNX1* is present (*red*, *arrowed*) along with non-rearranged *RUNX1* allele (larger *red spot*). Equivalent pre-leukaemic cells also detected in healthy co-twins of patients with *BCR-ABL1* ALL and hyperdiploid ALL

unselected, frozen cord blood samples also provided evidence for a pre-natal origin of *ETV6-RUNX1* fusions [36] (Fig. 4.3). Approximately 1 % of cord bloods in a series of ~600 collected and stored as viable cells for clinical (transplantation) were

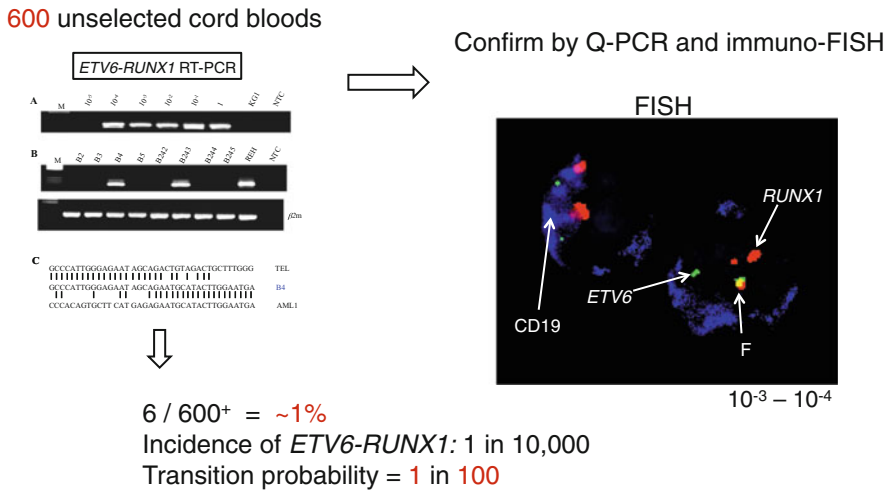

Fig. 4.5 t(12;21) ETV6-RUNX1$^+$ cells in normal cord blood (Data taken from ref. [36])

found to have expanded populations of B cell precursors with t(12;21) detected by multi-colour fluorescence, *in situ* hybridisation and by RT-PCR and Q-PCR (for mRNA) [36] (Fig. 4.5).

The cells are present at ~10^{-4} of circulating B cells suggesting that *ETV6-RUNX1* is a relatively weak oncogene endowing only minimal clonal or fitness advantage (see further below). One percent of cord bloods is some 100× the incidence rate of *ETV6-RUNX1*$^+$ ALL suggesting that the pre-leukaemic clones with this fusion are generated at a relatively high rate in normal foetal development, but have a low penetrance of disease either because they do not persist or because of a low probability (~1 %) of acquiring the necessary secondary events.

The maximum ages of children presenting with *ETV6-RUNX1*$^+$ ALL that have fusion gene-positive neonatal blood spots (– 9 years) or who share a common *in utero* origin with a co-twin (– 14 years) testifies to the protracted, covert latency of *ETV6-RUNX1* pre-malignant clones initiated before birth [37]. Pre-malignant clones with *ETV6-RUNX1* may persist at diagnosis, survive chemotherapy and give rise to very late relapses [38].

4.5 *ETV6-RUNX1* Fusion as an Initiating Event or Founder Mutation in ALL

Chromosome translocations are often assumed to be early or initiating events in the molecular pathogenesis of leukaemia and other cancers, but evidence endorsing this view is limited. In the case of the t(12;21), *ETV6-RUNX1* translocation and fusion there is good evidence that it is indeed the primary or initiating event in ALL that carry this marker. These data are summarised in Table 4.1.

Table 4.1 t(12;21), ETV6-RUNX1 fusion as the initiating or founder genetic event in childhood ALL

1.	The fusion is the only recurrent 'driver' genetic event shared (but non-constitutive) in monozygotic twins with concordant ALL [23, 27]
2.	All individual leukaemic cells analysed by multi-colour FISH or single cell Q-PCR have the fusion gene [38, 39]. In contrast, other genetic abnormalities are sub-clonally segregated in the branching phylogenetic tree of clonal evolution in each patient
3.	The translocation and fusion gene is retained in all cases that relapse [38]
4.	*ETV6-RUNX1* can transform mouse [40–44] and human stem cells [28] into a pre-leukaemic state which, in the presence of additional genetic abnormalities or genotoxic insults (in murine models), evolves to overt ALL

In addition to the twin studies referred to above, clonal phylogeny data support the contention that *ETV6-RUNX1* fusion is a founder lesion. Interrogation of the composite genetics of hundreds of single cells from individual patients by multi-colour FISH [39] or Q-PCR [45] can be used to construct clonal evolutionary trees or phylogenies. These reveal that *ETV6-RUNX1* is in every leukaemic cell, including those that reside at the base or trunk of the evolutionary tree. Similar studies in renal cancer using multi-regional sequencing to infer clonal phylogeny place VHL mutations as the likely common founder event for that cancer [46]. It is likely, though not formally proven, that *KMT2A*/*MLL* fusions in infant ALL and *BCR-ABL1* in ALL are also initiating genetic events. They are retained in relapse and, in the case of *BCR-ABL1*$^+$ ALL, are present in the trunk or base of the clonal phylogenetic tree (M-J Carnicer and MG, unpublished observations).

Functional evidence that *ETV6-RUNX1* (Table 4.1) or other fusion genes can initiate leukaemogenesis in model systems accords with a founder role in the clinical disease but by itself is not unambiguous evidence. *NOTCH1* mutations are highly prevalent in T-ALL [47] but in some patients, they appear to be sub-clonal [48]. In model systems, *Notch1* mutation can initiate T-ALL [49] or be secondarily acquired during the progression of leukaemia initiated by other mutations [50].

4.6 Transforming and Maintenance Functions of ETV6-RUNX1 Protein

Both ETV6 and RUNX1 are transcription factors that play a critical role in normal haematopoiesis. It is likely, therefore, that the chimaeric ETV6-RUNX1 protein initiates ALL and maintains the longevity of initiated clones via an altered pattern of transcriptional regulation. Widely expressed in human tissues, ETV6 is a member of the *ETS* (E-26 transforming) family of transcription factors that contain a C-terminal sequence-specific DNA binding domain which can also mediate interaction with other proteins. Binding of ETV6 to DNA is normally strongly repressed by an auto inhibitory mechanism that requires a conformational change in a C-terminal inhibitory domain to stimulate DNA binding [51]. Homo- and

hetero-dimerization of ETV6, through the highly conserved N-terminal 'pointed' (PTD) or helix-loop-helix domain, also serves to facilitate the DNA binding process [52]. A central repression domain is involved in transcriptional repression of ETV6 target sequences through the recruitment of co-repressor complexes including NCOR1, SIN3A and HDAC's [53, 54]. Chimeric knockout mice show that *ETV6* is required for normal bone marrow haematopoiesis but not for that in foetal liver [55].

RUNX1 is a member of the RUNT homology domain (RHD) family of transcription factors and is also widely expressed throughout haematopoiesis where it acts as a critical master switch [56]. *RUNX1* knockout models uncover its role in the formation of the definitive stem cell and in definitive bone marrow haematopoiesis [57, 58]. Aside from the DNA binding RHD domain, RUNX1 contains a transactivation domain, three inhibitory domains, a proline/serine/threonine-rich domain and a nuclear matrix targeting signal region [59, 60]. RUNX1 can itself bind weakly to DNA but its affinity for binding is increased by means of a non-DNA-binding hetero-dimerization partner, CBFB [61, 62]. In addition, dimerization of CBFB to RUNX1 protects RUNX1 from rapid proteasome degradation [63]. Recruitment of other regulatory transcription factors such as CEBPA, SPI1/PU.1 and PAX5, via adjacent DNA binding sites in RUNX1 target genes, also leads to enhanced RUNX1 DNA binding [63–65]. A variety of post-translational modifications can regulate RUNX1 activity [66] and alter lineage-specific gene expression through protein-protein interactions with co-activators (such as EP300 and CREBBP) or co-repressors (such as SIN3A and TLE1) [67]. These interactions reveal a context dependent role for RUNX1 as either an activator or repressor of transcription.

In ALL, the translocation t(12;21) results in an in-frame fusion between the first five exons of ETV6 and almost the entire coding region of RUNX1; bringing together the PTD and repression domains of ETV6 and the DNA binding, repressor and transactivation domains of RUNX 1 [12, 13] (Fig. 4.6).

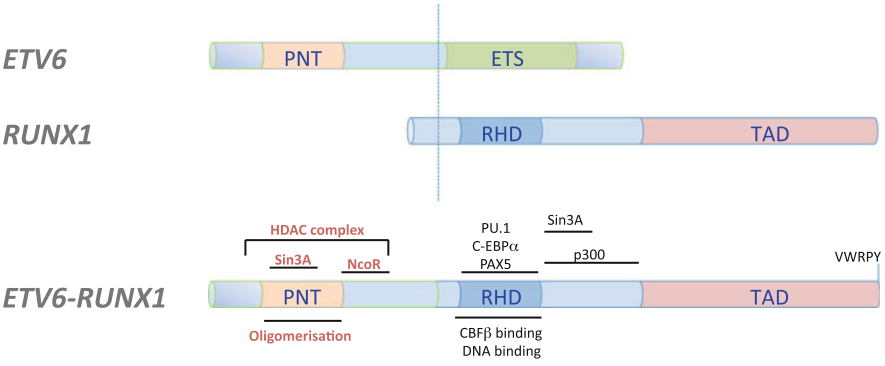

Fig. 4.6 Functional domains in ETV6-RUNX1 fusion. A schematic representation of the full-length ETV6, RUNX1 and ETV6-RUNX1 proteins. The fusion gene retains the oligomerisation (*PTD*) and repression domains of ETV6 and the DNA binding (*RHD*), repressor and transactivation domains of RUNX1. Regions reported to interact with various activator and co-repressor proteins mentioned in the text are also shown along with a Carboxy-terminal VWRPY motif that can bind Groucho-related corepressors

Both the RUNT domain and CBFB have been shown to be necessary for ETV6-RUNX1 activity [62, 68] and the retained PTD domain is required to allow dimerization with either ETV6-RUNX1 itself or with the normal ETV6 protein [40]. Similarly, RUNX1 activity can be regulated by homo-dimerization that may play a role in chromatin remodelling [69]. Accordingly, ETV6-RUNX1 can exert its effect on multiple signalling pathways.

Impaired B cell differentiation from the pro-B to the pre-B cell stage has been observed in a number of studies performed using retroviral transduction of *ETV6-RUNX1* into bone marrow stem cells followed by transplantation into syngeneic mice [40, 41, 70]. In contrast, retroviral transduction of *ETV6-RUNX1* into foetal liver cells affords the persistent self-renewal of pro-B cells both *in vitro* and *in vivo* [42, 71]. Levels of expression of the fusion gene were shown to be critical to outcome; low level expression of ETV6-RUNX1 promoted self-renewal of pro-B cells, whereas high level expression promoted self-renewal of myeloid cells [71]. Similarly, a perturbation of haematopoietic stem cell (HSC) numbers and an impairment of B cell differentiation have been identified in adult mice using either an ETV6-RUNX1 transgenic [72] or knock-in approach [43]. Schindler and colleagues show the disruption in HSC's to be present in adult haematopoiesis but not in foetal liver and therefore suggest that the pre-leukaemic clone in humans is disposed to emerge *in utero*, while its emergence in adults would be impaired [43]. Lentiviral delivery of *ETV6-RUNX1* into human $CD34^+$ cord blood cells, either grown on MS5 feeder layers or allowed to reconstitute the bone marrow of irradiated NOD-SCID mice, gives rise to an expanded compartment of pro-B cells not seen in normal human peripheral blood or bone marrow [28, 72]. These cells possess altered self-renewal properties and carry the 'pre-leukaemic' phenotype $CD34^+CD38^{-/low}$ $CD19^+$ [28].

Transient transfection studies confirm that ETV6-RUNX1 can inhibit RUNX1-dependent transcription at a number of RUNX1 target genes (e.g., CSF1R/MCSFR, TRB) and suggest that the ETV6 repression domains acquired in the fusion convert RUNX1 from an activator to a repressor [53, 73].

The signal pathways that are pivotal in transformation and maintenance of pre-leukaemic clones are not entirely resolved but candidates have been identified. The fusion protein has been shown to interfere with apoptosis, affecting anti-apoptotic genes such as survivin, [74] and has been proposed to act as a dominant negative transcription factor that can reduce expression of tumour suppressor genes while increasing expression of anti-apoptotic genes [75], although this does not seem to be the case in murine HSC's [43]. MicroRNAs (miRNAs) regulate the post-transcriptional expression level of a variety of genes by binding to complementary sequences located mainly in 3′-untranslated regions of target messenger RNA [76]. miRNA-494 has been identified as a direct miRNA target of *ETV6-RUNX1* and, along with miRNA-320a, was found to be up-regulated upon ETV6-RUNX1 silencing [77]. These miRNAs are expressed at low levels in *ETV6-RUNX1*$^+$ leukaemias and are observed to block expression of survivin, suggesting that miRNA suppression by ETV6-RUNX1 may also play a role in pre-leukaemic cell survival. In contrast, miR-125b has been associated with *ETV6-RUNX1*$^+$ ALL in patients, where its overexpression confers a survival advantage [78].

TGFB1 is a negative regulator of human early B cell progenitors [79] and also has a well-recognised role in cancer progression [80]. Data from three model systems provides evidence that ETV6-RUNX1 is able to compromise the TGFB1 signalling pathway and contribute both to the maintenance of covert pre-leukaemic clones and their competitive positive selection in an inflammatory context [72].

The erythropoietin receptor gene (*EPOR*) appears to be consistently highly expressed, ectopically, in *ETV6-RUNX1*$^+$ ALL [81–83]. Recent evidence suggests that ETV6-RUNX1 can directly regulate expression of *EPOR* [84] and promote expression of a functional cell surface receptor that is capable of binding its ligand; erythropoietin (EPO). Activation of JAK-STAT signalling via the EPOR:EPO axis and promotion of cell survival has also been confirmed in both murine and human models of ETV6-RUNX1 pre-leukaemia [84]. Whether the survival of *ETV6-RUNX1*$^+$ pre-leukaemic clones in patients is sustained by EPO is at present unknown.

These data provide some insight into how *ETV6-RUNX1* promotes the survival of pre-leukaemic clones but do not explain the partial differentiation arrest in the B cell precursor level. One possibility is that the major attrition of normal B precursor cells that have non-functional *IGH* rearrangements is blocked.

The question remains as to whether the ETV6-RUNX1 protein remains indispensable for clonal maintenance once the secondary mutations are accrued and clinically overt ALL emerges. Two separate studies that used RNAi to knockdown expression of the fusion gene in leukaemia cell lines have provided conflicting results. Zaliova and colleagues [85] used siRNA specific to the fusion gene and reported no differences in cell cycle arrest, apoptosis or attenuation of clonogenic potential. In contrast, Fuka [86] and co-workers used shRNA to show that suppression of ETV6-RUNX1 expression abrogates proliferation and cell survival and inhibits signalling via the PIK3CA/PI3K pathway. The impaired repopulation capacity of silenced ETV6-RUNX1 cell lines in xeno-transplants provided further evidence that ETV6-RUNX1 may indeed be indispensable for leukaemia maintenance [86].

4.7 Secondary Mutations that Complement *ETV6-RUNX1* Fusion

The observations on concordance rates of *ETV6-RUNX1*$^+$ ALL in monozygotic twins and the presence of ‘stalled’ evolution of pre-leukaemic clones in healthy co-twins of patients with ALL [28] and in unselected cord bloods all indicate that the t(12;21) translocation is insufficient for overt or malignant ALL, as does experimental modelling in mice [40–44]. The necessary additional genetic events have now been described. The structural rearrangements and coding region sequence changes that complement *ETV6-RUNX1* and drive clonal evolution have been identified by paired end and exomic sequencing in a series of 57 cases [24]. The total mutation load is relatively small as in most cases of different leukaemic subtypes and most paediatric cancers – compared with epithelial carcinomas [2]. Two

mutational signatures were identified. The most recurrent secondary genetic events are CNA, most of which are deletions. Almost 50 % of these have complete or partial nonamer-heptamer RAG recognition motifs. In contrast, in several thousand CNA in breast, pancreatic and prostate cancers, none had RAG recognition sequences at or near (<20 bp) the breakpoints. These data provide compelling evidence that critical secondary events that complement *ETV6-RUNX1* fusion in ALL are instigated by off-target RAG activity. Single nucleotide variants are less recurrent in *ETV6-RUNX1*$^+$ ALL, with the exception of *WHSC1*/*NSD2* [87], but there are, on average, ~14 per case, several or most of which could be 'passenger' mutations [24]. The predominant mutational signatures for these SNV were transitions or transversions at cytosines [24] indicative of a role of APOBEC enzymes in cytosine deamination as in other cancers [88]. In contrast to high risk subtypes of childhood ALL [89], *ETV6-RUNX1*$^+$ ALL have very few mutations impinging on kinase pathways, which may help explain the very good prognosis [24].

4.8 The Contextual Role of *ETV6-RUNX1* Fusion in the Clonal Evolution of ALL

The genetic and functional data now available on *ETV6-RUNX1*$^+$ ALL suggest a model for the role the chromosome translocation and fusion gene encoded protein play in the step-wise clonal evolution of ALL. This is summarised in Fig. 4.7. The exact cell type of origin of the t(12;21) is uncertain. Modelling studies with murine and human cells suggests that it could be a lympho-myeloid stem cell [28, 40–44].

If this is the case, then the fusion may bias differentiation into the B lineage. The major functional impact appears to be to generate a long-lived pre-leukaemic clone that proliferates slowly, in partial differentiation arrest in the B precursor compartment and with minimal clonal advantage in the absence of additional genetic alterations. The retention or slow passage of cells in the normally very transient and pro-apoptotic B precursor compartment where RAG/AICDA mediated *IG* rearrangements are ongoing may render them vulnerable to RAG/AICDA/APOBEC mediated structural changes in other genes [90]. Epidemiological evidence suggests that infections may act, indirectly, as a promotional factor in this process [91], possibly via the selective expansion of pre-leukaemic clones elicited by TGFB1 [72]. It is striking that the most highly recurrent secondary genetic changes in *ETV6-RUNX1*$^+$ ALL are predominantly deletions in genes that encode B cell differentiation promoting transcription factors or the cell cycle negative regulator CDKN2A/p16 [24, 92]. As a consequence of these secondary genetic events, leukaemic cells are then trapped in the precursor compartment, proliferating with the exit routes of apoptosis or differentiation no longer available.

> Now, here, you see, it takes all the running you can do, to keep in the same place. (The Red Queen to Alice in *Through the Looking Glass* by Lewis Carroll)

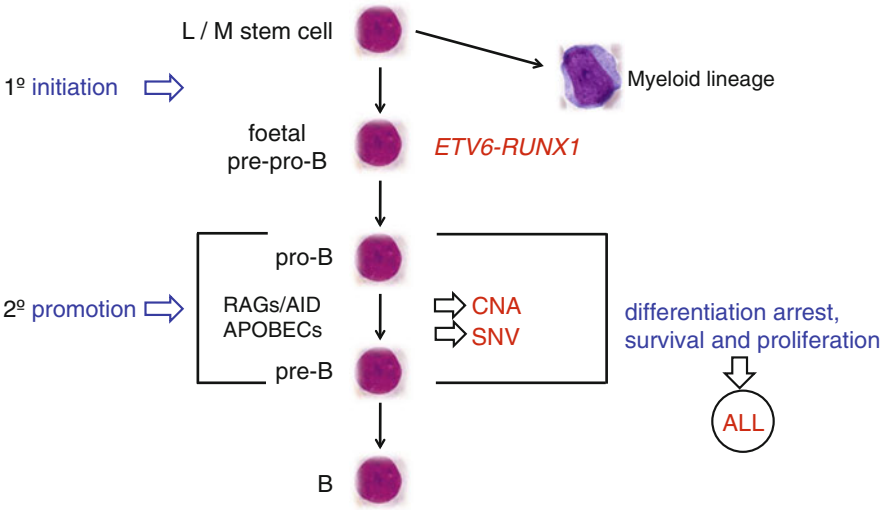

Fig. 4.7 Step-wise clonal evolution in ETV6-RUNX1⁺ ALL

4.9 Other Translocations as Initiating Lesions in Leukaemia and Cancer

The available data are compatible with the notion that several of the major chromosomal translocations in acute leukaemia of childhood are probably initiating or founding genetic lesions. This includes *ETV6-RUNX1*, *KMT2A/MLL* fusions (in infants), *BCR-ABL1* and *RUNX1-RUNX1T1* (in AML). In adult leukaemia, *PML-RARA* fusion in acute promyelocytic leukaemia (APML) is another strong candidate founder lesion [93], as is *BCR-ABL1* in CML [94]. The fact that therapeutic targeting of these latter two lesions is a very effective treatment strategy [93, 95] (albeit with recurrence due to resistance mutations in some cases) may be at least in part due to the fact that all leukaemic cells express the target.

The range of biochemical or signalling functions exercised by these translocation generated gene fusions indicate that there is unlikely to be a universal mechanism of cellular transformation and cancer/leukaemia initiation. Furthermore, it is clear that genetic alterations other than chromosome translocations can in all likelihood initiate acute leukaemia including chromosomal trisomies in ALL [96, 97], *DMN3TA* in AML [98] and a variety of mutations in myelodysplastic syndromes [99].

Outside of the leukaemias, other highly recurrent fusion genes [100] are good candidates as founder lesions, including *EWSR1-FLI1* fusions in Ewing's sarcomas [101], *TTYH1-C19MC* in a subset of embryonal neuroectodermal tumours [102], *IGH* translocations in multiple myeloma and its precursor lesion *MGUS* [103] and *TMPRSS2-ERG* fusions in prostate cancer [104, 105]. Detailed single cell genetics and phylogenetic tree assembly might consolidate their status and highlight their potential for both monitoring residual disease and as therapeutic targets.

References

1. Greaves M, Maley CC (2012) Clonal evolution in cancer. Nature 481:306–313
2. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA Jr, Kinzler KW (2013) Cancer genome landscapes. Science 339:1546–1558
3. Greaves M (2000) Cancer. The evolutionary legacy. Oxford University Press, Oxford
4. Domazet-Lošo T, Tautz D (2010) Phylostratigraphic tracking of cancer genes suggests a link to the emergence of multicellularity in metazoa. BMC Biol 8:66
5. Rowley JD (2001) Chromosome translocations: dangerous liaisons revisited. Nat Rev Cancer 1:245–250
6. Rabbitts TH (1994) Chromosomal translocations in human cancer. Nature 372:143–149
7. Aman P (ed) (2005) Fusion oncogenes-transformation mechanisms and tumor type specificity. Semin Cancer Biol 15(3)
8. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144(5):646–674
9. Ferrando AA, Look AT (2000) Clinical implications of recurring chromosomal and associated molecular abnormalities in acute lymphoblastic leukemia. Semin Hematol 37(4):381–395
10. Pui C-H, Relling MV, Downing JR (2004) Acute lymphoblastic leukemia. N Engl J Med 350:1535–1548
11. Romana SP, Le Coniat M, Berger R (1994) t(12;21): a new recurrent translocation in acute lymphoblastic leukemia. Genes Chromosomes Cancer 9(3):186–191
12. Romana SP, Mauchauffe M, Le Coniat M, Chumakov I, Le Paslier D, Berger R et al (1995) The t(12;21) of acute lymphoblastic leukemia results in a tel-AML1 gene fusion. Blood 85(12):3662–3670
13. Golub TR, Barker GF, Bohlander SK, Hiebert SW, Ward DC, Bray-Ward P et al (1995) Fusion of the *TEL* gene on 12p13 to the *AML1* gene on 21q22 in acute lymphoblastic leukemia. Proc Natl Acad Sci U S A 92:4917–4921
14. Shurtleff SA, Buijs A, Behm FG, Rubnitz JE, Raimondi SC, Hancock ML et al (1995) *TEL*/*AML1* fusion resulting from a cryptic t(12;21) is the most common genetic lesion in pediatric ALL and defines a subgroup of patients with an excellent prognosis. Leukemia 9:1985–1989
15. Seeger K, Adams H-P, Buchwald D, Beyermann B, Kremens B, Niemeyer C et al (1998) *TEL-AML1* fusion transcript in relapsed childhood acute lymphoblastic leukemia. Blood 91:1716–1722
16. Bhojwani D, Pei D, Sandlund JT, Jeha S, Ribeiro RC, Rubnitz JE et al (2012) ETV6-RUNX1-positive childhood acute lymphoblastic leukemia: improved outcome with contemporary therapy. Leukemia 26(2):265–270
17. Fielding AK, Rowe JM, Buck G, Foroni L, Gerrard G, Litzow MR et al (2014) UKALLXII/ECOG2993: addition of imatinib to a standard treatment regimen enhances long-term outcomes in Philadelphia positive acute lymphoblastic leukemia. Blood 123(6):843–850
18. Nussenzweig A, Nussenzweig MC (2010) Origin of chromosomal translocations in lymphoid cancer. Cell 141(1):27–38
19. Tsai AG, Lu H, Raghavan SC, Muschen M, Hsieh C-L, Lieber MR (2008) Human chromosomal translocations at CpG sites and a theoretical basis for their lineage and stage specificity. Cell 135:1130–1142
20. Wiemels JL, Greaves M (1999) Structure and possible mechanisms of *TEL-AML1* gene fusions in childhood acute lymphoblastic leukemia. Cancer Res 59:4075–4082
21. Greaves MF, Wiemels J (2003) Origins of chromosome translocations in childhood leukaemia. Nat Rev Cancer 3:639–649
22. Greaves MF, Maia AT, Wiemels JL, Ford AM (2003) Leukemia in twins: lessons in natural history. Blood 102:2321–2333

23. Ma Y, Dobbins SE, Sherborne AL, Chubb D, Galbiati M, Cazzaniga G et al (2013) Developmental timing of mutations revealed by whole-genome sequencing of twins with acute lymphoblastic leukemia. Proc Natl Acad Sci U S A 110(8):7429–7433
24. Papaemmanuil E, Rapado I, Li Y, Potter NE, Wedge DC, Tubio J et al (2014) RAG-mediated recombination is the predominant driver of oncogenic rearrangement in *ETV6-RUNX1* acute lymphoblastic leukemia. Nat Genet 46(2):116–125
25. Berger MF, Lawrence MS, Demichelis F, Drier Y, Cibulskis K, Sivachenko AY et al (2011) The genomic complexity of primary human prostate cancer. Nature 470(7333):214–220
26. Ford AM, Bennett CA, Price CM, Bruin MCA, Van Wering ER, Greaves M (1998) Fetal origins of the *TEL-AML1* fusion gene in identical twins with leukemia. Proc Natl Acad Sci U S A 95:4584–4588
27. Bateman CM, Colman SM, Chaplin T, Young BD, Eden TO, Bhakta M et al (2010) Acquisition of genome-wide copy number alterations in monozygotic twins with acute lymphoblastic leukemia. Blood 115(17):3553–3558
28. Hong D, Gupta R, Ancliffe P, Atzberger A, Brown J, Soneji S et al (2008) Initiating and cancer-propagating cells in *TEL-AML1*-associated childhood leukemia. Science 319:336–339
29. Alpar D, Wren D, Ermini L, Mansur MB, van Delft FW, Bateman CM, Titley I, Kearney L, Szczepanski T, Gonzalez D, Ford AM, Potter NE, Greaves M (2015) Clonal origins of ETV6-RUNX1+ acute lymphoblastic leukemia: studies in monozygotic twins. Leukemia 29:839–846
30. Ford AM, Ridge SA, Cabrera ME, Mahmoud H, Steel CM, Chan LC et al (1993) *In utero* rearrangements in the trithorax-related oncogene in infant leukaemias. Nature 363:358–360
31. Cazzaniga G, van Delft FW, Lo Nigro L, Ford AM, Score J, Iacobucci I et al (2011) Developmental origins and effect of *BCR-ABL1* fusion and *IKZF1* deletions in monozygotic twins with Ph+ acute lymphoblastic leukemia. Blood 118(20):5559–5564
32. Dobbins SE, Sherborne AL, Ma YP, Bardini M, Biondi A, Cazzaniga G et al (2013) The silent mutational landscape of infant MLL-AF4 pro-B acute lymphoblastic leukemia. Genes Chromosomes Cancer 52(10):954–960
33. Wiemels JL, Cazzaniga G, Daniotti M, Eden OB, Addison GM, Masera G et al (1999) Prenatal origin of acute lymphoblastic leukaemia in children. Lancet 354:1499–1503
34. Gale KB, Ford AM, Repp R, Borkhardt A, Keller C, Eden OB et al (1997) Backtracking leukemia to birth: identification of clonotypic gene fusion sequences in neonatal blood spots. Proc Natl Acad Sci U S A 94:13950–13954
35. Wiemels JL, Xiao Z, Buffler PA, Maia AT, Ma X, Dicks BM et al (2002) In utero origin of t(8;21) *AML1-ETO* translocations in childhood acute myeloid leukemia. Blood 99:3801–3805
36. Mori H, Colman SM, Xiao Z, Ford AM, Healy LE, Donaldson C et al (2002) Chromosome translocations and covert leukemic clones are generated during normal fetal development. Proc Natl Acad Sci U S A 99:8242–8247
37. Maia AT, Koechling J, Corbett R, Metzler M, Wiemels JL, Greaves M (2004) Protracted postnatal natural histories in childhood leukemia. Genes Chromosomes Cancer 39:335–340
38. van Delft FW, Horsley S, Colman S, Anderson K, Bateman C, Kempski H et al (2011) Clonal origins of relapse in *ETV6-RUNX1* acute lymphoblastic leukemia. Blood 117:6247–6254
39. Anderson K, Lutz C, van Delft FW, Bateman CM, Guo Y, Colman SM et al (2011) Genetic variegation of clonal architecture and propagating cells in leukaemia. Nature 469:356–361
40. Fischer M, Schwieger M, Horn S, Niebuhr B, Ford A, Roscher S et al (2005) Defining the oncogenic function of the TEL/AML1 (*ETV6*/*RUNX1*) fusion protein in a mouse model. Oncogene 24:7579–7591
41. Tsuzuki S, Seto M, Greaves M, Enver T (2004) Modelling first-hit functions of the t(12;21) *TEL-AML1* translocation in mice. Proc Natl Acad Sci U S A 101:8443–8448

42. Morrow M, Horton S, Kioussis D, Brady HJM, Williams O (2004) TEL-AML1 promotes development of specific hematopoietic lineages consistent with preleukemic activity. Blood 103:3890–3896
43. Schindler JW, Van Buren D, Foudi A, Krejci O, Qin J, Orkin SH et al (2009) TEL-AML1 corrupts hematopoietic stem cells to persist in the bone marrow and initiate leukemia. Cell Stem Cell 5(1):43–53
44. van der Weyden L, Giotopoulos G, Rust AG, Matheson LS, van Delft FW, Kong J et al (2011) Modeling the evolution of *ETV6-RUNX1*-induced B-cell precursor acute lymphoblastic leukemia in mice. Blood 118:1041–1051
45. Potter NE, Ermini L, Papaemmanuil E, Cazzaniga G, Vijayaraghavan G, Titley I et al (2013) Single-cell mutational profiling and clonal phylogeny in cancer. Genome Res 23(12):2115–2125
46. Gerlinger M, Horswell S, Larkin J, Rowan AJ, Salm MP, Varela I et al (2014) Genomic architecture and evolution of clear cell renal cell carcinomas defined by multiregion sequencing. Nat Genet 46:225–233
47. Grabher C, von Boehmer H, Look AT (2006) Notch 1 activation in the molecular pathogenesis of T-cell acute lymphoblastic leukaemia. Nat Rev Cancer 6(5):347–359
48. Mansour MR, Duke V, Foroni L, Patel B, Allen CG, Ancliff PJ et al (2007) Notch-1 mutations are secondary events in some patients with T-cell acute lymphoblastic leukemia. Clin Cancer Res 13(23):6964–6969
49. Chen J, Jette C, Kanki JP, Aster JC, Look AT, Griffin JD (2007) NOTCH1-induced T-cell leukemia in transgenic zebrafish. Leukemia 21(3):462–471
50. Lin YW, Nichols RA, Letterio JJ, Aplan PD (2006) Notch1 mutations are important for leukemic transformation in murine models of precursor-T leukemia/lymphoma. Blood 107(6):2540–2543
51. De S, Chan AC, Coyne HJ 3rd, Bhachech N, Hermsdorf U, Okon M et al (2014) Steric mechanism of auto-inhibitory regulation of specific and non-specific DNA binding by the ETS transcriptional repressor ETV6. J Mol Biol 426(7):1390–1406
52. Green SM, Coyne HJ 3rd, McIntosh LP, Graves BJ (2010) DNA binding by the ETS protein TEL (ETV6) is regulated by autoinhibition and self-association. J Biol Chem 285(24):18496–18504
53. Hiebert SW, Sun W, Davis JN, Golub T, Shurtleff S, Buijs A et al (1996) The t(12;21) translocation converts AML-1B from an activator to a repressor of transcription. Mol Cell Biol 16(4):1349–1355
54. Guidez F, Petrie K, Ford AM, Lu H, Bennett CA, MacGregor A et al (2000) Recruitment of the nuclear receptor corepressor N-CoR by the TEL moiety of the childhood leukemia-associated TEL-AML1 oncoprotein. Blood 96:2557–2561
55. Wang LC, Swat W, Fujiwara Y, Davidson L, Visvader J, Kuo F et al (1998) The *TEL*/*ETV6* gene is required specifically for hematopoiesis in the bone marrow. Genes Dev 12:2392–2402
56. Downing JR (2001) AML1/CBFbeta transcription complex: its role in normal hematopoiesis and leukemia. Leukemia 15(4):664–665
57. Okuda T, van Deursen J, Hiebert SW, Grosveld G, Downing JR (1996) AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. Cell 84:321–330
58. Wang Q, Stacy T, Binder M, Marin-Padilla M, Sharpe AH, Speck NA (1996) Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. Proc Natl Acad Sci U S A 93(8):3444–3449
59. Kitabayashi I, Yokoyama A, Shimizu K, Ohki M (1998) Interaction and functional cooperation of the leukemia-associated factors AML1 and p300 in myeloid cell differentiation. EMBO J 17(11):2994–3004

60. Zeng C, van Wijnen AJ, Stein JL, Meyers S, Sun W, Shopland L et al (1997) Identification of a nuclear matrix targeting signal in the leukemia and bone-related AML/CBF-alpha transcription factors. Proc Natl Acad Sci U S A 94(13):6746–6751
61. Kagoshima H, Shigesada K, Satake M, Ito Y, Miyoshi H, Ohki M et al (1993) The Runt domain identifies a new family of heteromeric transcriptional regulators. Trends Genet 9(10):338–341
62. Morrow M, Samanta A, Kioussis D, Brady HJ, Williams O (2007) TEL-AML1 preleukemic activity requires the DNA binding domain of AML1 and the dimerization and corepressor binding domains of TEL. Oncogene 26(30):4404–4414
63. Huang G, Shigesada K, Ito K, Wee HJ, Yokomizo T, Ito Y (2001) Dimerization with PEBP2beta protects RUNX1/AML1 from ubiquitin-proteasome-mediated degradation. EMBO J 20(4):723–733
64. Smith LT, Hohaus S, Gonzalez DA, Dziennis SE, Tenen DG (1996) PU.1 (Spi-1) and C/EBP alpha regulate the granulocyte colony-stimulating factor receptor promoter in myeloid cells. Blood 88(4):1234–1247
65. Huang G, Zhang P, Hirai H, Elf S, Yan X, Chen Z et al (2008) PU.1 is a major downstream target of AML1 (RUNX1) in adult mouse hematopoiesis. Nat Genet 40(1):51–60
66. Wang L, Huang G, Zhao X, Hatlen MA, Vu L, Liu F et al (2009) Post-translational modifications of Runx1 regulate its activity in the cell. Blood Cells Mol Dis 43(1):30–34
67. Yamagata T, Maki K, Mitani K (2005) Runx1/AML1 in normal and abnormal hematopoiesis. Int J Hematol 82(1):1–8
68. Roudaia L, Cheney MD, Manuylova E, Chen W, Morrow M, Park S et al (2009) CBFbeta is critical for AML1-ETO and TEL-AML1 activity. Blood 113(13):3070–3079
69. Li D, Sinha KK, Hay MA, Rinaldi CR, Sauntharajah Y, Nucifora G (2007) RUNX1-RUNX1 homodimerization modulates RUNX1 activity and function. J Biol Chem 282(18):13542–13551
70. Bernardin F, Yang Y, Cleaves R, Zahurak M, Cheng L, Civin CI et al (2002) TEL-AML1, expressed from t(12;21) in human acute lymphocytic leukemia, induces acute leukemia in mice. Cancer Res 62:3904–3908
71. Tsuzuki S, Seto M (2013) TEL (ETV6)-AML1 (RUNX1) initiates self-renewing fetal pro-B cells in association with a transcriptional program shared with embryonic stem cells in mice. Stem Cells 31(2):236–247
72. Ford AM, Palmi C, Bueno C, Hong D, Cardus P, Knight D et al (2009) The *TEL-AML1* leukemia fusion gene dysregulates the TGFb pathway in early B lineage progenitor cells. J Clin Invest 119(4):826–836
73. Fears S, Gavin M, Zhang DE, Hetherington C, Ben-David Y, Rowley JD et al (1997) Functional characterization of ETV6 and ETV6/CBFA2 in the regulation of the MCSFR proximal promoter. Proc Natl Acad Sci U S A 94(5):1949–1954
74. Diakos C, Krapf G, Gerner C, Inthal A, Lemberger C, Ban J et al (2007) RNAi-mediated silencing of TEL/AML1 reveals a heat-shock protein- and survivin-dependent mechanism for survival. Blood 109(6):2607–2610
75. Krug U, Ganser A, Koeffler HP (2002) Tumor suppressor genes in normal and malignant hematopoiesis. Oncogene 21(21):3475–3495
76. Shivdasani RA (2006) MicroRNAs: regulators of gene expression and cell differentiation. Blood 108(12):3646–3653
77. Diakos C, Zhong S, Xiao Y, Zhou M, Vasconcelos GM, Krapf G et al (2010) TEL-AML1 regulation of survivin and apoptosis via miRNA-494 and miRNA-320a. Blood 116(23):4885–4893
78. Gefen N, Binder V, Zaliova M, Linka Y, Morrow M, Novosel A et al (2010) Hsa-mir-125b-2 is highly expressed in childhood ETV6/RUNX1 (TEL/AML1) leukemias and confers survival advantage to growth inhibitory signals independent of p53. Leukemia 24(1):89–96
79. Ichii M, Oritani K, Yokota T, Nishida M, Takahashi I, Shirogane T et al (2008) Regulation of human B lymphopoiesis by the transforming growth factor-b superfamily in a newly estab-

lished coculture system using human mesenchymal stem cells as a supportive microenvironment. Exp Hematol 36:587–597
80. Massagué J, Blain SW, Lo RS (2000) TGFb signaling in growth control, cancer, and heritable disorders. Cell 103:295–309
81. Fine BM, Stanulla M, Schrappe M, Ho M, Viehmann S, Harbott J et al (2004) Gene expression patterns associated with recurrent chromosomal translocations in acute lymphoblastic leukemia. Blood 103(3):1043–1049
82. Inthal A, Krapf G, Beck D, Joas R, Kauer MO, Orel L et al (2008) Role of the erythropoietin receptor in ETV6/RUNX1-positive acute lymphoblastic leukemia. Clin Cancer Res 14(22):7196–7204
83. van Delft FW, Bellotti T, Luo Z, Jones KL, Patel N, Yiannikouris O et al (2005) Prospective gene expression analysis accurately subtypes acute leukaemia in children and establishes a commonality between hyperdiploidy and t(12;21) in acute lymphoblastic leukaemia. Br J Haematol 130:26–35
84. Torrano V, Procter J, Cardus P, Greaves M, Ford AM (2011) ETV6-RUNX1 promotes survival of early B lineage progenitor cells via a dysregulated erythropoietin receptor. Blood 118(18):4910–4918
85. Zaliova M, Madzo J, Cario G, Trka J (2011) Revealing the role of TEL/AML1 for leukemic cell survival by RNAi-mediated silencing. Leukemia 25(2):313–320
86. Fuka G, Kantner HP, Grausenburger R, Inthal A, Bauer E, Krapf G et al (2012) Silencing of ETV6/RUNX1 abrogates PI3K/AKT/mTOR signaling and impairs reconstitution of leukemia in xenografts. Leukemia 26(5):927–933
87. Jaffe JD, Wang Y, Chan HM, Zhang J, Huether R, Kryukov GV et al (2013) Global chromatin profiling reveals NSD2 mutations in pediatric acute lymphoblastic leukemia. Nat Genet 45(11):1386–1391
88. Roberts SA, Lawrence MS, Klimczak LJ, Grimm SA, Fargo D, Stojanov P et al (2013) An APOBEC cytidine deaminase mutagenesis pattern is widespread in human cancers. Nat Genet 45(9):970–976
89. Roberts KG, Morin RD, Zhang J, Hirst M, Zhao Y, Su X et al (2012) Genetic alterations activating kinase and cytokine receptor signaling in high-risk acute lymphoblastic leukemia. Cancer Cell 22(2):153–166
90. Swaminathan S, Klemm L, Park E, Papaemmanuil E, Ford A, Kweon S-M, Trageser D, Hasselfeld B, Henke N, Mooster J, Geng H, Schwarz K, Kogan SC, Casellas R, Schatz DG, Lieber MR, Greaves MF, Müschen M (2015) Mechanisms of clonal evolution in childhood acute lymphoblastic leukemia. Nat Immunol 16:766-774
91. Greaves M (2006) Infection, immune responses and the aetiology of childhood leukaemia. Nat Rev Cancer 6:193–203
92. Mullighan CG, Goorha S, Radtke I, Miller CB, Coustan-Smith E, Dalton JD et al (2007) Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. Nature 446:758–764
93. de The H, Chen Z (2010) Acute promyelocytic leukaemia: novel insights into the mechanisms of cure. Nat Rev Cancer 10(11):775–783
94. Melo JV, Barnes DJ (2007) Chronic myeloid leukaemia as a model of disease evolution in human cancer. Nat Rev Cancer 7(6):441–453
95. Sawyers CL (2009) Shifting paradigms: the seeds of oncogene addiction. Nat Med 15:1158–1161
96. Lane AA, Chapuy B, Lin CY, Tivey T, Li H, Townsend EC et al (2014) Triplication of a 21q22 region contributes to B cell transformation through HMGN1 overexpression and loss of histone H3 Lys27 trimethylation. Nat Genet 20
97. Bateman CM, Alpar D, Ford AM, Colman SM, Wren D, Morgan M, Kearney L, Greaves M (2015) Evolutionary trajectories of hyperdiploid ALL in monozygotic twins. Leukemia 29:58–65

98. Shlush LI, Zandi S, Mitchell A, Chen WC, Brandwein JM, Gupta V et al (2014) Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. Nature 506(7488):328–333
99. Papaemmanuil E, Gerstung M, Malcovati L, Tauro S, Gundem G, Van Loo P et al (2013) Clinical and biological implications of driver mutations in myelodysplastic syndromes. Blood 122(22):3616–3627; quiz 3699
100. Mitelman F, Johansson B, Mertens F (2007) The impact of translocations and gene fusions on cancer causation. Nat Rev Cancer 7(4):233–245
101. Riggi N, Cironi L, Provero P, Suva ML, Kaloulis K, Garcia-Echeverria C et al (2005) Development of Ewing's sarcoma from primary bone marrow-derived mesenchymal progenitor cells. Cancer Res 65(24):11459–11468
102. Kleinman CL, Gerges N, Papillon-Cavanagh S, Sin-Chan P, Pramatarova A, Quang DA et al (2014) Fusion of TTYH1 with the C19MC microRNA cluster drives expression of a brain-specific DNMT3B isoform in the embryonal brain tumor ETMR. Nat Genet 46(1):39–44
103. Morgan GJ, Walker BA, Davies FE (2012) The genetic architecture of multiple myeloma. Nat Rev Cancer 12(5):335–348
104. Clark J, Attard G, Jhavar S, Flohr P, Reid A, De-Bono J et al (2008) Complex patterns of ETS gene alteration arise during cancer development in the human prostate. Oncogene 27(14):1993–2003
105. Braun M, Menon R, Nikolov P, Perner S (2010) *ERG* rearrangement as a clonal expansion marker for prostate cancer. Open Pros Cancer J 3:63–68

Chapter 5
Common Chromosomal Fragile Sites and Cancer

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Contents

5.1 Historical Aspects and Characteristics of Fragile Sites 74
5.2 Mechanisms of Fragile Site Expression 77
5.2.1 Brief Overview of DNA Replication 77
5.2.2 Replication Dynamics of the Common Fragile Sites 78
5.2.2.1 Characteristics of the Inducers of Fragile Site Expression 78
5.2.2.2 Replication Dynamics of FRA3B and FRA16D 79
5.2.2.3 Replication Dynamics of Other Common Fragile Sites 80
5.2.2.4 Possible Mechanism(s) Linking DNA Replication and Fragile Site Expression 81
5.2.3 Other Classes of Fragile Sites (Early-Replicating AID-Independent) 84
5.3 Relationship of Fragile Sites to Cancer 85
5.3.1 Mechanisms Leading to Common Fragile Site Expression in Cancer 85
5.3.1.1 Oncogene-Induced DNA Replication Stress 85
5.3.1.2 Mutations in Checkpoint and DNA Repair Pathways 86
5.3.1.3 Aberrant Epigenetic States 86
5.3.2 Role of Fragile Sites in Chromosomal Alterations in Cancer 87
5.3.2.1 Inactivation of Tumour Suppressor Genes by Deletion 87
5.3.2.2 Overexpression of Oncogenes by Amplification 87
5.3.2.3 Deregulation of Genes via Chromosomal Translocations 88
5.3.2.4 Integration of Viral DNA Sequences 88
5.3.2.5 New Potential Cancer-Specific Fragile Sites 89
5.4 Future Directions and Unanswered Questions 89
Acknowledgements 90
References 90

Abstract Chromosomal fragile sites are specific loci that show gaps, breaks, or rearrangements in metaphase chromosomes when cells are cultured under conditions that partially inhibit DNA synthesis, inducing replication stress. The common fragile sites (CFS) are numerous in the human genome, and are essentially observed in all individuals. Although the molecular basis for chromosome fragility of CFSs

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remains incompletely understood, there is now general agreement that CFS sequences have a distinct replication programme that combines late replication with failure to activate origins in the core regions of the CFSs during replication stress, resulting in the failure to complete replication. The CFSs exhibit several features characteristic of highly unstable or recombinogenic regions of the genome, and CFSs have been shown to mediate genetic instability in cancers, including during the early stages of tumourigenesis. In this chapter, we review the molecular features of CFSs, as well as the relationship of CFSs to genomic alterations in cancer cells.

Keywords Chromosomal fragile sites • Common fragile sites • DNA replication • Genetic instability • Mechanism of instability

5.1 Historical Aspects and Characteristics of Fragile Sites

Chromosomal fragile sites are specific loci that show gaps, breaks, or rearrangements in metaphase chromosomes when cells are cultured under particular conditions that partially inhibit DNA synthesis [1, 2]. Fragile sites are grouped into two major classes based upon their frequency in the population, as well as the culture conditions required for their expression. The "rare fragile sites" number at least 30, and are found in less than 5 % of the population, in many instances, in only one or a few families. A number of rare fragile sites, including the FRAXA, FRAXE, FRAXF, FRA16A, FRA16B, and FRA11B, have been cloned or mapped at the molecular level (reviewed in [1]). With the exception of the FRA16B, the mutation leading to the expression of fragility is the expansion and methylation of a CGG trinucleotide repeat, and chromosome breakage occurs within this small segment of DNA. FRA16B also involves the expansion of a repeat – 33 bp AT-rich minisatellite. Expansion of these repeats can give rise to genetic disease by modifying the expression of genes in which they are located, as in the case of the FRAXA and FRAXF, or by mediating chromosomal deletions, as seen in some cases of Jacobsen Syndrome [1].

In contrast, numerous "common fragile sites" (CFSs) have been recognized – 87 CFSs are listed in the NCBI database (http://www.ncbi.nlm.nih.gov/gene/?term=%28common+fragile+site %29+AND+%22Homo+sapiens%22[porgn%3A__txid9606]). However, the precise number depends on the inducing conditions, cell type, and analytical methods; a recent study reported 230 CFSs, although most of these sites were expressed very infrequently [3]. As described below, the greater the impairment of replication, the more CFSs observed, until the cessation of replication. The expression of CFSs varies in different cell types, but the CFSs are essentially observed in all individuals [4, 5]. At least 45 common fragile sites have now been mapped at the sequence level, including the most frequently expressed sites: FRA3B, FRA6F,

FRA7G, FRA7F, FRA16D, FRAXB (reviewed in [6]). Molecular analysis has provided evidence that the CFSs differ from the rare fragile sites in several ways. First, the CFSs span large genomic regions, ranging from 160 kb to greater than 10 Mb, and genomic breakage and instability occurs over a large region (reviewed in [2, 6, 7]). Second, despite extensive analysis of several CFSs, no specific sequence elements or repeat motifs, such as the trinucleotide repeats characteristic of rare fragile sites, have been identified to be required for their expression (reviewed in [2, 7]). Common fragile sites are conserved, and have also been observed in many other mammalian species, such as mouse, hamster, primates, dogs, cattle, and deer mouse. Furthermore, at least eight mouse CFSs have human CFS orthologs: Fra14A2 (FRA3B), Fra8E (FRA16D), Fra6C1 (FRA4F), Fra12C1 (FRA7K), Fra2D (FRA2G), Fra6A3.1 (FRA7G), Fra6C1 (FRA7H), and Fra4C2 (FRA9E), and regions orthologous to the human FRA3B/*FHIT* and FRA16D/*WWOX* are conserved in *Mus musculus* [8]. In yeast, chromosome breaks at specific sites called "replication slow zones" have been proposed to be analogous to CFSs [9]. Thus, fragile sites appear to be maintained across species, although their function is unknown.

The common fragile sites exhibit several features characteristic of highly unstable or recombinogenic regions of the genome. In addition to forming breaks and gaps on metaphase chromosomes, they are preferred sites for sister chromatid exchanges (SCEs), chromosomal deletions and rearrangements, the integration of transfected plasmid DNA or viruses, e.g., HPV, and the initiation of breakage-fusion-bridge (BFB) cycles, leading to gene amplification [2, 6, 7]. CFSs have also been shown to be preferred sites for structural variation in stem cells [10], and copy number variants in the human germline [11]. Recent studies have shed light on the role of CFSs in genetic instability in cancer cells. For example, Bignell et al. demonstrated that a substantial proportion of homozygous and hemizygous deletions in cancer cells cluster in CFSs [12]. The compendium of CFSs principally consists of large regions containing genes >300 kb in length, and over half of the recurrent molecular deletions in cancer cells originate in CFSs that are associated with large genes.

At present, the molecular basis for chromosome fragility of CFSs remains incompletely understood (Fig. 5.1). Local genomic features, including G-negative chromosomal bands distal to centromeres, enrichment for *ALU* repeats, high DNA flexibility, CpG island density, transcription start site density, H3K4me1 coverage, and mononucleotide microsatellite coverage are significant predictors [6, 7, 13]. We first demonstrated that CFSs replicate late in S-phase, and sometimes remain incompletely replicated in metaphase cells, [14] and there is now general agreement that CFSs remain incompletely replicated at the onset of mitosis following replication stress, making them prone to breakage. Moreover, CFS instability is dependent on ATR signalling and is associated with other DNA damage response factors [2]. For the past decade, several nonexclusive models have existed for CFS instability. The first model posits that CFSs contain sequences that are difficult to replicate, leading to stalled replication forks and, ultimately, replication fork collapse. The second model suggests that CFSs contain a paucity of replication origins, resulting in incomplete replication under replication stress. Recent studies of eight of the major CFSs have resulted in a convergence of both models revealing that a distinct

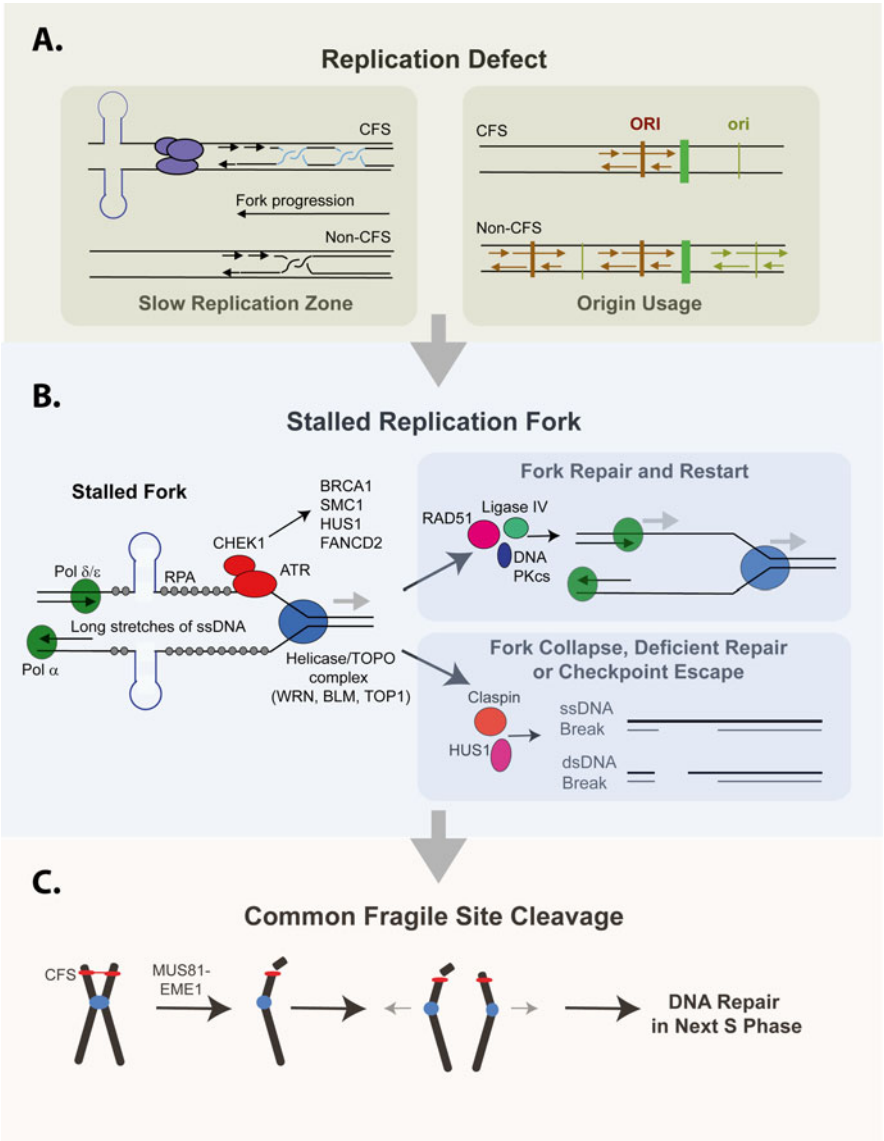

Fig. 5.1 Model for the induction of common fragile sites. The model predicts that CFSs have a distinct replication programme that combines late replication with failure to activate origins in the core regions of the CFSs following replication stress, ultimately leading to long stretches of ssDNA. (**a**) *Left panel*. Common fragile sites could represent slow replicating regions as a result of an unusual chromatin structure, the presence of bulky DNA-protein complexes hindering replication fork progression, or persistence of post-replicative structures in the presence of APH. In this event, origins may initiate replication in early- to mid-S phase, but replication continues into late S phase. *Right panel*. CFSs have been shown to have an unusual distribution of primary and secondary origins – a lower density of primary origins at fragile sites may prevent completion of replication in the presence of APH within the S phase. A lower density of secondary origins, or lack of initiation at the secondary origins, may prevent rescue of replication by these inefficient origins when the primary origin is stalled (*Green bar*) or slowed in the presence of APH leading to

replication programme combining late replication with failure to activate origins in the core regions of the CFSs following replication stress is responsible for the failure to complete replication. Because replication programmes differ in various cell types, different repertoires of expressed CFSs are found in human cells [6]. In this chapter, we review the features of DNA replication of common fragile sites, and the role of replication in the genetic instability characteristic of these sequences, as well as the relationship of CFSs to genomic alterations in cancer cells.

5.2 Mechanisms of Fragile Site Expression

5.2.1 Brief Overview of DNA Replication

DNA replication in eukaryotes initiates at specific sites called origins of replication [15]. In *Saccharomyces cerevisiae*, origins of replication, known as Autonomously Replicating Sequence (ARS) share an A/T rich, 11 bp ARS consensus sequence that is recognized by the origin recognition complex (ORC) proteins. In contrast, the identification of metazoan origins of replication has proven to be much more difficult. Over the past two decades, a number of approaches have been undertaken to define metazoan origins, including low-throughput methods, e.g., two-dimensional gel electrophoresis techniques or nascent strand abundance assays and, more recently, genome-scale approaches that are combined with microarray or sequencing technologies (reviewed in [16, 17]). There is a notable low level of reproducibility between laboratories and across methods, especially in the case of the genome-wide techniques, suggesting that both cell intrinsic, i.e., only a subset of the active origins of any particular cell population have been mapped, and cell extrinsic, i.e., the subset of origins identified is method-dependent, factors are involved (reviewed in [16, 17]). Nevertheless, these methods have demonstrated that, despite the evolutionary conservation of the replication machinery, metazoan origins do not have the sequence specificity observed in *S. cerevisiae* – rather, they may be defined by DNA structure, such as G-quadruplex-forming DNA motifs [18].

Fig. 5.1 (continued) unreplicated regions within fragile sites. (**b**) *Left panel*. The ssDNA binding protein, RPA, coats the resultant unreplicated ssDNA and recruits the DNA damage response checkpoint proteins, including ATR, which activate S-phase or G_2/M checkpoints. *Right panel*. Repair of these regions mediated by RAD51 and PRKDC (DNA-PKcs) and other proteins promotes replication fork progression. Some CFS sequences may escape checkpoint activation or are left unrepaired, resulting in an unreplicated region in G_2/M. **c** MUS81-EME1 is recruited to such sites in prophase or early metaphase, and cleaves any remaining replication forks at CFSs (*red circles* represent FANCD2 foci at CFSs) to permit the sister chromatids to be disjoined in anaphase, giving rise to the characteristic cytological appearance of chromosome breaks/gaps at metaphase. Thereafter, the unreplicated DNA is repaired in the subsequent S phase. Repair of DNA breaks can result in molecular deletions or structural chromosomal rearrangements involving CFSs, which have been identified in cancers (Figure modified from references, [2, 38, 77])

The molecular mechanism of replication initiation is a highly conserved and tightly regulated process in all eukaryotes (reviewed in [19]). The first step involves licensing of origins in the late M or early G_1 phase by the assembly of pre-replicative complexes (pre-RCs) at non-active origins as well as at the active origins, which includes ORC1-6, CDT1, CDC6, and minichromosome maintenance (MCM) 2-7 complex. Using genome-wide origin mapping approaches, metazoan genomes were found to have a very large number of origins, up to one every 11 kb, only a subset of which are activated in any given cell within a population [16, 18].

The second step corresponds to the loading of CDC45, which is triggered by two kinases, cyclin-CDK and CDC7-DBF4. The cyclin-CDK complex leads to progression of cells into S phase, and CDC7-DBF4 leads to activation of origin-firing by phosphorylation of the MCM proteins. Origin activation is followed by initiation of DNA replication by loading of the single-stranded DNA (ssDNA) binding protein, Replication Protein A [20], and the primase-DNA polymerase complex. The bidirectional replication fork is now active and can move into the elongation phase. In higher eukaryotes, the origins are not synchronously activated at the onset of the S phase; rather, they follow a precise and reproducible sequence of initiation throughout S phase (reviewed in [21]). Although not completely understood, this temporal replication programme has been linked to multiple biological factors: GC content, LINE (Long Interspersed Nuclear Elements) density, gene density, transcriptional activity, chromatin structure and, more recently, with large-scale chromatin folding (reviewed in [21]). For instance, transcriptionally active, GC-rich euchromatin tends to replicate before the condensed, silent, and GC-poor heterochromatin. As described later, the integrity of DNA replication is monitored during S phase by checkpoint proteins [22]. If replication is stalled or the DNA template damaged, the checkpoint proteins arrest the cell in S phase, and prevent entry into G_2 until the fork is restored or the damage repaired.

5.2.2 Replication Dynamics of the Common Fragile Sites

5.2.2.1 Characteristics of the Inducers of Fragile Site Expression

The majority of the CFSs are induced by aphidicolin (APH) and, less frequently, bromo-2′deoxyuridine (BrdU), 5-azacytidine (5-Aza-C), 5-fluorouracil and camptothecin (reviewed in [2]), chemicals that interfere with DNA replication. Moreover, fragile site expression requires induction during the preceding S phase [14]. APH is an antibiotic, which inhibits DNA polymerases α, δ, and ε by competing with the incorporation of dCTP and, to a lesser extent, dTTP. High doses of APH (from 15 to 300 μM) block DNA elongation very rapidly and trigger an intra-S checkpoint, blocking cell cycle progression in early S phase and preventing initiation at late replicating domains [23]. At the lower doses of APH used for CFS induction (0.2–0.8 μM), cells still progress through S phase, but do so much more slowly than in an unperturbed S phase [24]. BrdU is incorporated into DNA in place of thymidine; at

high concentrations of BrdU, S-phase progression is blocked [25]. 5-Aza-C, an inhibitor of DNA methyltransferases, inhibits chromatin condensation within G bands and heterochromatin (late-replicating) and, perhaps as a direct consequence, advances the replication timing of late-replicating chromosomal regions [26].

As mentioned above, the nature of the fragile site-inducing agents suggested that fragile site expression was likely to involve DNA synthesis. DNA repair mechanisms may also play a role, since caffeine, an inhibitor of the G_2 checkpoint, increases the number of cells expressing CFSs. These observations, together with the high frequency of SCEs [27] and chromosome rearrangements at CFSs, led investigators to propose a number of years ago that fragile sites were associated with unreplicated DNA or DNA strand breaks.

5.2.2.2 Replication Dynamics of FRA3B and FRA16D

The FRA3B, at 3p14.2, lies within the Fragile Histidine Triad (*FHIT*) gene, and is the most highly expressed CFS in lymphoblastoid cells when DNA replication is perturbed by APH [4, 28]. The *FHIT* gene spans 1.6 Mb, but encodes only a 1.1 kb transcript. Large intragenic deletions within the FRA3B sequences, have been identified in a variety of tumour cells [4, 28]. By analyzing the replication timing of FRA3B in peripheral blood lymphocytes and human Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines, we and others showed that FRA3B alleles replicate in late S phase in untreated cells [14, 24, 29]. Exposure to APH resulted in a small, but significant, delay in the timing of replication of the FRA3B alleles, and some cells entered mitosis without completing the replication of these sequences [14].

To elucidate the link between DNA replication and CFS expression, our laboratory mapped active origins in the *FHIT*/FRA3B locus in non-malignant lymphoblastoid cells, using two independent methods, a nascent strand DNA assay combined with microarray analysis developed in our laboratory [30] and chromatin immunoprecipitation targeting ORC and MCM proteins [24] and mapped 100 ± 22 origins within the 1.6 Mb region. Several of the origins that mapped within the FRA3B core were also identified in an independent analysis of another lymphoblastoid cell line using the bubble-trapping method combined with deep sequencing analysis [31]. We found that FRA3B had significantly fewer, smaller, and more widely-dispersed origins as compared to its flanking non-CFS sequences (Lucas et al. unpublished results). Using a DNA combing and FISH method, the Debatisse laboratory did not detect initiation events within the FRA3B core, suggesting that the FRA3B region is replicated by long-travelling forks coming from origins located outside of the FRA3B [32]. Nonetheless, the approach used did not exclude the presence of "low efficiency" origins within the FRA3B in comparison to surrounding non-fragile regions. Indeed, we observed significantly less newly-replicated DNA in untreated cells at origins located within FRA3B, as compared to those located in flanking, non-fragile regions, suggesting that CFS origins are less efficient and/or have a faster fork speed (Lucas et al. unpublished results) [24].

Furthermore, Letessier et al. demonstrated a direct correlation between DNA replication and expression of breakage at FRA3B in cells with differential levels of breakage. That is, low origin density and late completion of DNA replication in untreated cells were linked to high levels of CFS expression in APH-treated cells, whereas higher origin density and earlier replication were linked to low levels of breakage [32].

Taken together, these results suggest that, in lymphoblastoid cells under basal growth conditions, the FRA3B is characterized by a low density of weak origins in comparison to its flanking non-CFS sequences (Fig. 5.1) [24, 32, 33]. In the presence of APH, dormant origins fail to fire in the FRA3B region (Lucas et al. unpublished results), [32] strongly suggesting that FRA3B does not respond properly to replication stress [24, 32].

The FRA16D, at 16q23, is the second most highly-expressed CFS in human lymphoblastoid cells [4]. The boundaries of the genetically unstable sequences comprising the FRA16D span ~2.5 Mb, and include the *WWOX* gene, which spans ~1 Mb (reviewed in [34]). Large intragenic deletions within *WWOX* have been identified in a variety of tumours, including breast, esophageal, lung, ovarian, colon, and prostate carcinomas [34, 35]. Furthermore, it has been suggested that WWOX may function as a suppressor of tumour growth. Several laboratories have demonstrated that the FRA16D sequences replicate late in S phase and that, the entire FRA16D is contained within one or more late-replicating domains [24, 32, 34, 35].

5.2.2.3 Replication Dynamics of Other Common Fragile Sites

The FRA7H, at 7q32.3, spans a 161 kb region of intergenic DNA, that is 58 % AT-rich, and predicted to contain four regions of high flexibility [36]. Using FISH analysis of asynchronous human lymphoma cells, Hellman et al. showed that the FRA7H alleles initiate replication in the mid-S phase in an asynchronous manner with one allele replicating earlier than the other, without allelic specificity [37]. Furthermore, the FRA7H exhibited a bipolar gradient of replication, where replication initiates and occurs earlier at the centre of the 160 kb region than the adjacent regions on either side. APH delays replication at FRA7H and enhances the replication timing difference within the 160 kb region. Overall, these results suggest that the FRA7H region has intrinsic features that may delay replication.

The FRA7G, at 7q31.2, corresponds to an AT-rich (61 % AT-rich), 800 kb region that encompasses several genes (*TES*, *CAV1*, *CAV2*, and *MET*), and shows loss of heterozygosity (LOH) in several human malignancies [38]. *TES* (*TESTIN*) may represent a candidate tumour suppressor gene, and *MET* is amplified in many tumours. Hellman et al. showed that breaks at FRA7G in a gastric carcinoma cell line led to amplification of the *MET* gene by a BFB mechanism, providing further evidence for a role for CFSs in the amplification of oncogenes [39]. Although the absolute replication timing of FRA7G within S phase is unknown, Hellman et al., demonstrated that the FRA7G has a biallelic replication pattern, with one allele replicating late and the other one earlier, and that the replication fork(s) progress unusually slowly

within the fragile site [39]. At present, the effect of APH on the replication dynamics of FRA7G is unknown.

In two other CFSs, FRA1H and FRA2G, replication initiates during early to mid S-phase, but there is an intrinsic delay in replication progression and, by late S phase, approximately half of the CFS sequences remain unreplicated [40]. Using DNA combing techniques and FISH, Ozeri-Galai et al. determined that the FRA16C – which shares the same AT-rich genomic region as the FRA16B rare fragile site – is characterized by slow fork progression, and fork stalling at AT-rich sequences under basal conditions. Under replication stress, the frequency of fork stalling is exacerbated, and there is a failure to activate additional origins [41]. Finally, FRA6E – which contains the large 1.3 Mb *PARK2* gene – contains long AT-rich repeats across which replication is slowed [42]. Thus, CFS expression combines late and slow replication, increased replication fork arrest, and an apparent paucity of active origins leading to replication stress and instability.

5.2.2.4 Possible Mechanism(s) Linking DNA Replication and Fragile Site Expression

Slow Replication Domains and Replication Transition Zones

As described earlier for the FRA3B, CFSs may represent sequences that replicate very slowly under normal growth conditions, potentially due to a low density of less efficient origins, and that are unable to recover from a further delay in DNA synthesis following replication stress. The link between origin density/efficiency, slow completion of replication, and DNA breaks at CFSs was confirmed by comparing the replication dynamics and the frequency of breaks for several CFSs that show differential expression in two cell types – fibroblasts and lymphoblasts [32, 34]. Furthermore, CFS regions seem to represent transition zones between early and late replicating domains [43]. Interestingly, genome-wide mapping of the replication dynamics of the long arms of human chromosomes 11 and 21 by PCR amplification of flow sorted BrdU-labelled cells has shown that genes implicated in cancer and other diseases are significantly over-represented in the transition regions between early and late replication domains [44].

Stalled Replication Forks

APH inhibits replication fork elongation, leading to stalled forks. In this event, a convergent replication fork extending from a distant origin may complete replication, resulting in a delay in the process. Another potential consequence is the uncoupling of the DNA unwinding by the replicative helicase from the replication machinery, as observed in *Xenopus* egg extracts treated with APH during both the initiation and elongation steps, leading to the accumulation of ssDNA regions, and triggering the formation of abnormal structures [45]. Another consequence of the

replication machinery dissociation is that replication may not be able to resume, since some components (MCM2-7) can only be loaded onto the chromatin in the G_1 phase. Interestingly, these effects are only observed in cells with a mutation in the S phase checkpoint proteins Mec1p (ATM/ATR ortholog) or Rad53p (CHEK2), or RecQ helicase Sgs1p (BLM homolog).

In addition, CFSs could be more prone to form secondary DNA structures that are difficult to replicate, such as hairpins, or could lead to even more aberrant structures, when located near a stalled fork. Indeed, DNA sequence analysis of the FRA3B, FRA7G, FRA7H, and FRA16D revealed that the CFSs contain multiple regions that have the potential to form unusual DNA structures, including high flexibility, low stability, and non-B-forming sequences [36]. Similarly, as suggested by Cha and Kleckner, some regions of the genome could be preferential sites for the formation of DNA-protein complexes, which could hinder the passage of the replication fork [9].

Stalled replication forks or the presence of unreplicated DNA, may be converted to DSBs, and prolonged replication inhibition results in the accumulation of DSBs. Non-homologous end joining (NHEJ) and single strand annealing are employed by cells to process DSBs in the early cellular response [46]. These two pathways would not be expected to result in a visible fragile site lesion in the ensuing mitosis, but rather in deletion of fragile site sequences in one or both of the daughter cells. As DSBs accumulate, RAD51-mediated homologous recombination (HR) becomes the predominant mechanism of repair [47], a process that can result in formation of SCEs, as has been observed at CFSs. DSBs may also be repaired by ligation with homologous sequences from another chromosome, resulting in gross rearrangements, such as an unbalanced translocation, or they may be sites for ligation of exogenous DNA, e.g., viral sequences, as discussed later in Sect. 5.3.

Replication Defects at Fragile Sites and Checkpoints

In eukaryotic cells, the duplication of the genome during S phase and its transmission during G_2-M phase is monitored at multiple levels (reviewed in [19, 48]). Normal checkpoint mechanisms ensure that DNA replication occurs once, and only once, per cell cycle, and that mitosis does not begin until DNA replication is complete. The ssDNA present at stalled replication forks leads to recruitment of the ATR (Ataxia Telangiectasia Mutated- and Rad3-related) kinase, which, in turn, activates a variety of proteins, including the CHEK1 protein kinase. Phosphorylation by CHEK1 leads to sequestration of the CDC25C phosphatase in the cytoplasm, thereby abrogating activation of the mitotic CDK1 by dephosphorylation, and leading to cell cycle arrest in the S phase. Response to DSBs is mediated similarly by another checkpoint kinase, ATM (Ataxia Telangiectasia Mutated), leading to activation of CHEK2, and resulting in cell cycle arrest and DNA damage repair. However, a threshold level of unreplicated DNA may be required to activate the checkpoint(s), and very low levels of DNA replication very late in the cell cycle may not be sufficient to delay mitotic entry. Sequences with impaired replication progression, or

that replicate very late, would have a shorter period of time for DNA repair before the onset of mitosis. Unreplicated regions of DNA could affect localized chromatin structure, and manifest the recombinogenic properties of CFSs. In cultured cells challenged with APH or other CFS inducers, a fraction of cells escape the ATR replication checkpoint via a poorly understood mechanism, despite sustaining replication defects (stalled forks, aberrant replication structures, unreplicated DNA regions, etc.) at fragile site sequences. Moreover, fragile site induction is exacerbated in human cells in the absence of ATR or downstream targets, such as BRCA1, the Fanconi anaemia proteins, SMC1A/B, and CHEK1, indicating that the fragile site sequences are monitored by checkpoints, but sometimes escape [2, 9].

Transcription and Replication at CFSs

In bacteria and yeast, collisions of transcription complexes with moving replication forks cause genetic instability. To avoid this phenomenon, replication and transcription are spatially and temporally coordinated in eukaryotic cells. Helmrich et al. analyzed five CFSs associated with large genes, and found that the time required to transcribe genes >800 kb spans more than a single cell cycle, and that the long genes replicate late, regardless of their transcriptional activity. Regions of concomitant transcription and replication in late S phase lead to collisions of transcriptional machinery with replication forks, creating R-loops (RNA:DNA hybrids) resulting in breakage at CFSs, such as the FRA3B embedded within the >1.6 Mb *FHIT* gene, and the FRA16D within the 1.1 Mb *WWOX* gene [49]. In contrast, the results of other reports are not consistent with these findings. Le Tallec et al., observed plasticity in the location of the breaks within CFSs in different cell types, suggesting that transcription units per se do not set the borders of CFSs [34], and Jiang et al. found that the level of expression of the FRA3B was unrelated to the expression of *FHIT* in several lymphoid cell lines [50]. Moreover, this mechanism is unlikely to explain the fragility of all CFSs, since a large fraction of CFSs are not associated with large genes. Additional studies will be needed to clarify this relationship.

Chromatin Structure at CFSs

Epigenetically defined chromatin structure plays a critical role in the regulation of DNA replication and gene transcription. For example, open chromatin, characterized by the enrichment of active histone H3 acetylation marks, can facilitate origin firing during replication and lead to early replication during S phase [51]. Given that CFSs are late-replicating and manifest replication stress, Jiang et al. investigated whether chromatin conformation at CFSs plays a role in impaired DNA replication [50]. By using chromatin immunoprecipitation coupled with microarray analysis (ChIP-CHIP), the investigators mapped histone H3K9/14 acetylation (H3K9/14Ac) levels at the six most commonly expressed CFSs in EBV-transformed lymphoblastoid cells, and noted that the chromatin at CFSs was characterized by hypoacetylation as

compared to the surrounding, non-fragile DNA sequences. In addition, chromatin at the FRA3B was more resistant to micrococcal nuclease treatment, suggesting that CFS chromatin assumed a more condensed conformation. In this regard, treatment of the cells with the histone deacetylase inhibitor, Trichostatin A (TSA), reduced breakage at these CFSs, which was accompanied by an increase of H3K9/14Ac at these sites. Thus, this study linked chromatin conformation to genomic instability at CFSs, and established hypoacetylation as a characteristic epigenetic pattern of CFSs that may contribute to their defective response to replication stress.

5.2.3 Other Classes of Fragile Sites (Early-Replicating AID-Independent)

Recently, a different class of fragile sites was identified using genome-wide approaches [52]. Barlow et al. mapped early activating replication origins by Repli-Seq and RPA-associated ssDNA at stalled replication forks by ChIP-seq in synchronized early S phase B lymphocytes treated with hydroxyurea (HU), an inhibitor of ribonucleotide reductase that induces replication stress by the depletion of deoxynucleotide pools. Surprisingly, they observed a substantial overlap between the two sets of loci (nearly 80 %). Moreover, the majority of the RPA-bound sites were also marked with the DNA damage marker γ-H2AX and fork-repairing complex components, BRCA1 and SMC5, further confirming that these RPA-bound loci at early replication origins were sites of stalled and collapsed replication forks. In contrast, they did not detect similar DNA damage sites at known CFSs. To distinguish these sites of replication failure from canonical CFS that replicate in late-S phase, the authors designated these regions as Early Replicating Fragile Sites (ERFSs). The authors further demonstrated that DNA damage at ERFSs is ATR-dependent, but not activation-induced cytidine deaminase (AID)-dependent, suggesting that similar defects of DNA repair mechanisms may be involved in both ERFS and CFS expression. Moreover, oncogenic stress, such as *MYC* overexpression, triggers fragility at both ERFSs and CFSs and, like CFSs, ERFSs are often embedded within genomic regions that are deleted or amplified in cancers. Despite many similarities between ERFSs and CFSs, these two classes of fragile sites differ in several ways. First, ERFSs are associated with early firing replication origins, whereas CFSs typically replicate late. Second, ERFS sequences are enriched for CpG dinucleotides, whereas CFS sequences are AT-rich. Third, ERFS loci contain a high density of activated origins, whereas those CFSs that have been mapped at high resolution have a low density of activated origins [24, 30, 32]. Fourth, ERFSs are often associated with promoters of highly transcribed genes that are characterized by open chromatin conformation; CFSs are embedded in introns of large genes with more condensed chromatin conformation [50]. Further studies are needed to elucidate the different mechanisms through which genomic instability arises from these two classes of fragile sites.

5.3 Relationship of Fragile Sites to Cancer

More than 30 years ago, fragile sites were implicated in the recurring chromosomal abnormalities in cancer. In 1984, Le Beau and Rowley reported an association between the chromosomal location of fragile site and the breakpoints of the recurring chromosomal abnormalities, including translocations, inversions, deletions, and amplification in leukaemias and lymphomas [53]. Many of these abnormalities target oncogenes, such as *MYB*, *MOS*, *MYC*, and *HRAS*, suggesting that fragile sites may act as predisposing factors for chromosomal rearrangements, particularly those involving genes known to induce malignant transformation. During the past few decades, new evidence has revealed that CFSs play a much broader role in inducing genetic instability in cancers. Chromosomal abnormalities involving CFSs have been shown to inactivate tumour suppressor genes, enhance oncogene expression, and facilitate the integration of viral sequences, which may result in further genotoxic stress and lead to selection of clones that eventually develop into a malignant disease. Herein, we discuss the potential mechanisms that lead to CFS expression in cancers, and their molecular consequences.

5.3.1 Mechanisms Leading to Common Fragile Site Expression in Cancer

5.3.1.1 Oncogene-Induced DNA Replication Stress

CFSs are induced experimentally in vitro by low doses of APH, a DNA polymerase inhibitor. Recently, Arlt et al. demonstrated that treatment with low doses of HU leads to the formation of de novo copy number variants (CNVs) in cultured fibroblasts, and that these CNVs resembled the characteristics of CFSs induced by APH [11]. As described earlier, HU induces replication stress through a different mechanism than APH, via the depletion of deoxyribonucleotide pools, thereby impeding replication fork progression [54]. Results from this study suggest that regardless of the source, replication stress is a causal factor of deleterious CNVs, especially within CFSs.

In cancers, oncogene activation can lead to DNA replication stress, increased CFS expression, and the subsequent induction of genomic aberrations in several ways (reviewed in Hills and Diffley [55]). First, deregulation of the TP53 and RB1/E2F pathways and overexpression of *MYC* or HPV *E7* leads to a reduction in licensing of replication origins. Given that some CFSs are either inherently origin-deficient or fail to activate secondary origins following replication fork stalling [24, 30, 41], reduced origin licensing could further enhance these deficiencies and lead to increased fork collapse and accumulation of unreplicated ssDNA within CFSs. Second, once replication initiates, overexpression of oncogenes, such as *CCNE*, HPV *E6* and *E7*, *MYC*, and *RAS* family genes can increase origin firing. This is

particular harmful to CFSs that are embedded within large genes, and could be more susceptible to replication interference by the transcriptional machinery, leading to collisions between replication forks and transcription complexes and, eventually, the formation of DSBs. Increased origin firing within these CFSs may increase the chance that such collisions occur within CFSs. Third, many prereplicative complexes (pre-RC) components, such as CDT1 and CDC6 can act as oncogenes, and are often upregulated in response to *RAS* gene and *CCNE* overexpression. These activated pre-RC components lead to origin re-licensing, and the subsequent depletion of deoxyribonucleotide pools, a form of replication stress that is similar to HU treatment, which is known to induce CFSs. Taken together, it is possible that the increased genomic alterations of CFS loci seen in cancer cells are due, in part, to the replication stress induced by overexpression of oncogenes.

5.3.1.2 Mutations in Checkpoint and DNA Repair Pathways

DNA replication checkpoints and DNA repair pathways play important roles in the surveillance of the DNA damage associated with CFS expression. Unreplicated ssDNA and DSBs induced from collapsed stalled replication forks at CFSs are recognized by checkpoint proteins, and DNA damage sensing enzymes, such as ATR and ATM, which in turn activates repair pathways, including NHEJ. CFS expression is elevated when components of these pathways are mutated or downregulated, including ATR, ATM, CHEK1, BRCA1, FANCD2, PRKDC (DNA-PK), WRN, and BLM (reviewed in [7]), that are frequently mutated in cancer. For example, a survey of mutations and copy number alterations of *ATR* in cBioPortal, an online database for Cancer Genomics (http://www.cbioportal.org/public-portal/), reveals that *ATR* is targeted by missense and nonsense mutations, and frame-shift indels in a number of cancers, including bladder, breast, colorectal, head and neck, lung, ovarian, pancreas, melanoma, stomach, thyroid, and uterine cancers [56, 57]. Moreover, the aggregate frequency of mutations within select genes encoding components of the DNA damage checkpoint and repair pathways (*ATR*, *ATM*, *BRCA1*, *CHEK1*, *FANCD2*, *RAD51*, *PRKDC*, *WRN*, *BLM*) ranges from 10 to 40 % in cancer, with the higher frequency in solid tumours. Therefore, defects of DNA damage checkpoints and DNA repair due to frequent mutations in cancer may facilitate the expression of CFSs and lead to the pronounced genomic instability seen in cancer cells.

5.3.1.3 Aberrant Epigenetic States

In addition to the genetic features of CFS, a potential link between the epigenetic chromatin structure and CFS expression has been established recently. Jiang et al. demonstrated that several of the most frequent CFSs, including FRA3B and FRA16D, are characterized by a more condensed chromatin conformation than their surrounding, non-fragile regions, due to the lack of active histone acetylation marks [50]. Treatment with TSA and/or 5-Aza-C reduced chromosomal breakage at

CFSs. Recently, mutations targeting epigenetic regulators have been identified in many types of cancers. For example, the majority of non-Hodgkin lymphomas carry mutations within the genes encoding KMT2D (an H3K4 methyltransferase), CREBBP and EP300 (histone and non-histone acetyltransferases), and EZH2 (H3K27 methyltransferase) (reviewed in [58]). In myeloid malignancies, enzymes that regulate DNA methylation (DNMT3A), and hydroxymethylation (IDH1, IDH2, TET2) are frequently mutated as well [59]. Similar phenomena are also observed in solid tumours (reviewed in [60]). Although most studies have focused on elucidating the consequence(s) of these epigenetic modifier mutations in the regulation of gene promoters, it is reasonable to predict that these mutations may target broader genomic regions, including CFS sequences, to establish an aberrant epigenetic landscape in cancers. For example, mutations in *CREBBP or EP300* may further exacerbate hypoacetylation of CFSs, resulting in increased breakage. Further studies on the epigenetic mechanisms of CFS expression, particularly in cancers, are needed to shed light on the role of epigenetic marks and genomic instability involving CFSs.

5.3.2 Role of Fragile Sites in Chromosomal Alterations in Cancer

5.3.2.1 Inactivation of Tumour Suppressor Genes by Deletion

CFS expression has long been associated with genomic instability in cancers, including the gain or loss of genetic material spanning CFS loci, and translocations involving CFSs [61]. These genetic alterations can lead to inactivation of tumour suppressor genes or ectopic overexpression of oncogenes. For example, the FRA3B is embedded within a large tumour suppressor gene, *FHIT*, that is frequently deleted in lung and breast cancer, as well as other carcinomas [62]. Although *Fhit*$^{-/-}$ KO mice exhibited only a marginal increase of tumourigenesis in response to various carcinogens, crossing these mice with other disease models, such as *Vhl*$^{-/-}$ KO or *Nit1*$^{-/-}$ KO animals, rendered full penetrance of tumour development (reviewed in [63]), suggesting a cooperative role for FHIT during tumourigenesis. Recently, Saldivar et al. showed that loss of *Fhit* expression in precancerous lesions initiates genomic instability that may eventually facilitate malignant transformation, linking alterations at CFSs to the origin of cancer genomic instability [64]. Other examples of tumour suppressor gene loss involving CFSs, include *WWOX* within the FRA16D, *PARK2* within the FRA6E, and *CAV1* and *TES* within the FRA7K [63].

5.3.2.2 Overexpression of Oncogenes by Amplification

In addition to the loss of genetic material involving CFSs, genomic amplification of the *MET* oncogene with boundaries within FRA7G sequences was observed in a gastric carcinoma cell line [39] and primary esophageal adenocarcinoma [65].

Amplification of the *MET* locus leads to overexpression of *MET*, resulting in a poor prognosis. By applying dual-colour FISH, Hellman et al. mapped the centromeric boundary of the amplified region within the FRA7G, and demonstrated that amplification of the *MET* locus via FRA7G breakage was organized in an inverted repeat fashion, as predicted by the BFB model [39]. They proposed that an initial break occurred at the telomeric end, and led to end-fusion of the sister chromatids; thereafter, ongoing replication stress might induce persistent FRA7G expression resulting in successive amplification and cycles of BFB [39]. In addition to FRA7G, FRA7I has also been implicated in duplication of the *PIP* gene via BFB cycles in human breast cancer [66]. However, an oncogenic role for *PIP* has yet to be established.

5.3.2.3 Deregulation of Genes via Chromosomal Translocations

In addition to the aberrations described above, CFSs have also been linked to the formation of chromosomal translocations in cancer. It is notable that FRA3B, the most commonly expressed CFS, was cloned by mapping the genomic sequences involved in the t(3;8)(p14.2;q24.1) noted in a family with hereditary renal cell carcinoma [67, 68]. This translocation disrupts *FHIT*, resulting in its inactivation. A similar phenomena was also observed for FRA16D, which was found to be involved in the recurring t(14;16)(q32.3;q23) in multiple myeloma (MM) [69]. This translocation not only results in a truncated allele of the tumour suppressor gene, *WWOX*, but also places the *MAF* oncogene near the *IGH* locus, resulting in enhanced *MAF* expression [69]. Exactly how genomic instability at CFSs mediates the formation of translocations is not fully understood. The t(14;16) may be mediated by the RAG1, RAG2, and AID (activation-induced cytidine deaminase) proteins, which normally participate in rearranging the B-cell immunoglobulin genes and T-cell receptor genes to increase the diversification of antibodies [70]. Indeed, by using a novel Translocation Capture Sequencing method, Klein et al. mapped chromosomal rearrangements in B lymphocytes and demonstrated that AID was responsible for many translocations involving *MYC* and *IGH* in B-cell lymphomas [71]. Determining whether CFSs, such as FRA16D, contain DNA sequences or chromatin structures that can be recognized by RAGs and AID requires further investigation. It has also been proposed that BFB cycles and NHEJ can induce chromosomal fusions [70]. Finally, DSBs resulting from collapsed replication forks within CFSs may be another potential source of translocations.

5.3.2.4 Integration of Viral DNA Sequences

Due to the high frequency of DSBs at CFS, they were predicted to be the preferred sites for the integration of foreign DNA. Indeed, Rassool et al. utilized this feature to clone the FRA3B by transfecting exogenous marker DNA into cells in which FRA3B expression was induced by APH, and observed preferential integration of

the marker DNA at the FRA3B locus [72]. In cancers, CFSs have been found to be the integration sites for viral DNA sequences. For example, human papillomavirus (HPV), the most important cancer-related virus, is preferentially integrated into CFSs in cervical cancer cells [73, 74]. Recent studies demonstrated that expression of the HPV16 *E6*/*E7* genes leads to replication stress by significantly decreasing the cellular nucleotide pools, raising the possibility that CFSs may be prone to increased expression in HPV infected cells, facilitating successive (and preferential) integration of viral sequences [75, 76].

5.3.2.5 New Potential Cancer-Specific Fragile Sites

The recent expanded efforts to map copy number alterations (CNAs) in a large cohort of tumours and the development of sophisticated bioinformatics analyses has led to new insights into the genomic alterations involving CFSs in cancer. Bignell et al. profiled the genotype status and CNAs in 746 publicly available cancer cell lines across multiple tissue types by using Affymetrix SNP6.0 arrays [12]. They detected large homozygous deletion (HD) clusters preferentially targeting recessive cancer genes (tumour suppressor genes) and CFS loci. In addition, they observed different structural signatures of HD clusters targeting recessive cancer genes and CFSs. That is, there was a threefold increase in homozygous deletions at known recessive cancer genes than hemizygous deletions, whereas there were 66 % more hemizygous deletions occurring at known CFSs than homozygous deletions. This suggests that there is a higher rate of DNA breakage within CFSs affecting one allele, some of which subsequently acquired other deletions in the remaining allele. Moreover, using this structural signature, the authors showed that the majority of the unclassified HD clusters had structural features of CFS loci, suggesting that there are potentially more CFSs that have not been identified or mapped precisely. In this regard, CFSs have largely been examined in lymphocytes. A recent study combining Repli-Seq with cytogenetic analysis found the distribution of CFSs in fibroblasts is quite different from that of lymphocytes [34]. This study further showed that over 50 % of recurrent cancer deletions originate from CFSs associated with large genes in different tissue types. Therefore, it is reasonable to predict that these unclassified HD clusters span CFSs that are specific to certain tissues, and have yet to be mapped.

5.4 Future Directions and Unanswered Questions

The application of new technologies has led to substantial advances in our understanding of the genomic characteristics of CFSs, and DNA replication patterns in these regions of the genome. Elucidating the molecular basis of CFSs and their inherent instability is important in that they provide a unique opportunity to examine the molecular events that follow certain types of replication stress, and how such

replication stress leads to genetic instability within the replication-sensitive CFS sequences, ultimately leading to deletions, translocations, and other genomic aberrations in cancer. In addition, their instability in the earliest stages of tumour development provides an opportunity to examine their link to cell cycle checkpoints and DNA repair pathways. However, a number of questions remain, and we outline a few of these here. For example, what is the full spectrum of replication patterns at CFSs, and its relationship to DNA repair and cell cycle checkpoints? Does interference between transcription and replication play a mechanistic role in the expression of some CFSs? In vivo, what cellular processes/pathways lead to replication stress and genomic instability in premalignant cells and in cancer cells? Are there additional genomic aberrations in cancer cells that are mediated by genomic instability at CFSs? Are there mechanistic parallels between genomic instability at ERFSs and CFSs? Do CFSs have a biological function, or conserved function?

With respect to the last point, whether CFSs have a biological role has been the subject of considerable speculation. The evolutionary conservation of CFSs in widespread phyla argues for a conserved function. Nonetheless, such conservation is counterintuitive, given the likelihood that genetically unstable sequences might be detrimental to survival and, thus, selected against during evolution. Durkin and Glover proposed that the inherent fragility of these regions might in and of itself serve a valuable biological function [2]. They posited that CFSs may be among the last sequences to replicate, thereby serving to signal to the cell that replication is complete. Cell cycle checkpoints would monitor these sites, blocking entry into mitosis until their replication was complete. Intriguing data from the Hickson laboratory challenge this view, and suggest that breakage at CFSs actually promotes genomic stability [77]. These investigators observed that the DNA structure-specific nuclease MUS81-EME1 localizes to CFS loci in early mitotic cells. In contrast to the prevailing view that CFSs result from chromatin breaks during chromosome condensation, they found that cleavage of replication forks at CFSs (presumably unreplicated DNA) is an active MUS81-EME1 process, that promotes faithful sister chromatid disjunction at anaphase – replication would then be completed in the daughter cells in the subsequent S phase, thereby preserving the integrity of the genome. Further studies are needed to evaluate this intriguing model, as well as to unravel the complexity of CFS instability, and it's relevance to the development and progression of cancer.

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References

1. Sutherland GR (2003) Rare fragile sites. Cytogenet Genome Res 100:77–84
2. Durkin SG, Glover TW (2007) Chromosome fragile sites. Annu Rev Genet 41:169–192
3. Mrasek K, Schoder C, Teichmann AC et al (2010) Global screening and extended nomenclature for 230 aphidicolin-inducible fragile sites, including 61 yet unreported ones. Int J Oncol 36:929–940

4. Glover TW, Berger C, Coyle J, Echo B (1984) DNA polymerase alpha inhibition by aphidicolin induces gaps and breaks at common fragile sites in human chromosomes. Hum Genet 67:136–142
5. Yunis JJ, Soreng AL, Bowe AE (1987) Fragile sites are targets of diverse mutagens and carcinogens. Oncogene 1:59–69
6. Debatisse M, Le Tallec B, Letessier A, Dutrillaux B, Brison O (2012) Common fragile sites: mechanisms of instability revisited. Trends Genet TIG 28:22–32
7. Ozeri-Galai E, Bester AC, Kerem B (2012) The complex basis underlying common fragile site instability in cancer. Trends Genet TIG 28:295–302
8. McAllister BF, Greenbaum IF (1997) How common are common fragile sites: variation of aphidicolin-induced chromosomal fragile sites in a population of the deer mouse (Peromyscus maniculatus). Hum Genet 100:182–188
9. Cha RS, Kleckner N (2002) ATR homolog Mec1 promotes fork progression, thus averting breaks in replication slow zones. Science 297:602–606
10. Hussein SM, Batada NN, Vuoristo S et al (2011) Copy number variation and selection during reprogramming to pluripotency. Nature 471:58–62
11. Arlt MF, Ozdemir AC, Birkeland SR, Wilson TE, Glover TW (2011) Hydroxyurea induces de novo copy number variants in human cells. Proc Natl Acad Sci U S A 108:17360–17365
12. Bignell GR, Greenman CD, Davies H et al (2010) Signatures of mutation and selection in the cancer genome. Nature 463:893–898
13. Fungtammasan A, Walsh E, Chiaromonte F, Eckert KA, Makova KD (2012) A genome-wide analysis of common fragile sites: what features determine chromosomal instability in the human genome? Genome Res 22:993–1005
14. Le Beau MM, Rassool FV, Neilly ME et al (1998) Replication of a common fragile site, FRA3B, occurs late in S phase and is delayed further upon induction: implications for the mechanism of fragile site induction. Hum Mol Genet 7:755–761
15. Bell SP, Dutta A (2002) DNA replication in eukaryotic cells. Annu Rev Biochem 71:333–374
16. Gilbert DM (2012) Replication origins run (ultra) deep. Nat Struct Mol Biol 19:740–742
17. Mechali M (2010) Eukaryotic DNA, replication origins: many choices for appropriate answers. Nat Rev Mol Cell Biol 11:728–738
18. Besnard E, Babled A, Lapasset L et al (2012) Unraveling cell type-specific and reprogrammable human replication origin signatures associated with G-quadruplex consensus motifs. Nat Struct Mol Biol 19:837–844
19. Lucas I, Raghuraman MK (2003) The dynamics of chromosome replication in yeast. Curr Top Dev Biol 55:1–73
20. Achille A, Biasi MO, Zamboni G et al (1996) Chromosome 7q allelic losses in pancreatic carcinoma. Cancer Res 56:3808–3813
21. Rhind N, Gilbert DM (2013) DNA replication timing. Cold Spring Harb Perspect Med 3:1–26
22. Machida Y, Dutta A (2005) Cellular checkpoint mechanisms monitoring proper initiation of DNA replication. J Biol Chem 280:6253–6256
23. Dimitrova DS, Gilbert DM (2000) Temporally coordinated assembly and disassembly of replication factories in the absence of DNA synthesis. Nat Cell Biol 2:686–694
24. Palakodeti A, Lucas I, Jiang Y et al (2010) Impaired replication dynamics at the FRA3B common fragile site. Hum Mol Genet 19:99–110
25. Drouin R, Lemieux N, Richer CL (1990) Analysis of DNA replication during S-phase by means of dynamic chromosome banding at high resolution. Chromosoma 99:273–280
26. Collins N, Poot RA, Kukimoto I, Garcia-Jimenez C, Dellaire G, Varga-Weisz PD (2002) An ACF1-ISWI chromatin-remodeling complex is required for DNA replication through heterochromatin. Nat Genet 32:627–632
27. Glover TW, Stein CK (1987) Induction of sister chromatid exchanges at common fragile sites. Am J Hum Genet 41:882–890
28. Huebner K, Garrison PN, Barnes LD, Croce CM (1998) The role of the FHIT/FRA3B locus in cancer. Annu Rev Genet 32:7–31

29. Wang L, Darling J, Zhang JS, Huang H, Liu W, Smith DI (1999) Allele-specific late replication and fragility of the most active common fragile site, FRA3B. Hum Mol Genet 8:431–437
30. Lucas I, Palakodeti A, Jiang Y et al (2007) High-throughput mapping of origins of replication in human cells. EMBO Rep 8:770–777
31. Mesner LD, Valsakumar V, Cieslik M, Pickin R, Hamlin JL, Bekiranov S (2013) Bubble-seq analysis of the human genome reveals distinct chromatin-mediated mechanisms for regulating early- and late-firing origins. Genome Res 23:1774–1788
32. Letessier A, Millot GA, Koundrioukoff S et al (2011) Cell-type-specific replication initiation programs set fragility of the FRA3B fragile site. Nature 470:120–123
33. Hansen RS, Thomas S, Sandstrom R et al (2010) Sequencing newly replicated DNA reveals widespread plasticity in human replication timing. Proc Natl Acad Sci U S A 107:139–144
34. Le Tallec B, Millot GA, Blin ME, Brison O, Dutrillaux B, Debatisse M (2013) Common fragile site profiling in epithelial and erythroid cells reveals that most recurrent cancer deletions lie in fragile sites hosting large genes. Cell Rep 4:420–428
35. Palakodeti A, Han Y, Jiang Y, Le Beau MM (2004) The role of late/slow replication of the FRA16D in common fragile site induction. Genes Chromosomes Cancer 39:71–76
36. Mishmar D, Rahat A, Scherer SW et al (1998) Molecular characterization of a common fragile site (FRA7H) on human chromosome 7 by the cloning of a simian virus 40 integration site. Proc Natl Acad Sci U S A 95:8141–8146
37. Hellman A, Rahat A, Scherer SW, Darvasi A, Tsui LC, Kerem B (2000) Replication delay along FRA7H, a common fragile site on human chromosome 7, leads to chromosomal instability. Mol Cell Biol 20:4420–4427
38. Lucas I, Palakodeti A, Le Beau MM (2007) The implications of DNA replication in common fragile site expression. In: Arrieta I, Penagarikano O, Télez M (eds) Fragile sites: new discoveries and changing perspectives. Nova Science Publishers, Hauppauge, pp 67–98
39. Hellman A, Zlotorynski E, Scherer SW et al (2002) A role for common fragile site induction in amplification of human oncogenes. Cancer Cell 1:89–97
40. Pelliccia F, Bosco N, Curatolo A, Rocchi A (2008) Replication timing of two human common fragile sites: FRA1H and FRA2G. Cytogenet Genome Res 121:196–200
41. Ozeri-Galai E, Lebofsky R, Rahat A, Bester AC, Bensimon A, Kerem B (2011) Failure of origin activation in response to fork stalling leads to chromosomal instability at fragile sites. Mol Cell 43:122–131
42. Palumbo E, Matricardi L, Tosoni E, Bensimon A, Russo A (2010) Replication dynamics at common fragile site FRA6E. Chromosoma 119:575–587
43. Debatisse M, El Achkar E, Dutrillaux B (2006) Common fragile sites nested at the interfaces of early and late-replicating chromosome bands: cis acting components of the G2/M checkpoint? Cell Cycle 5:578–581
44. Watanabe Y, Fujiyama A, Ichiba Y et al (2002) Chromosome-wide assessment of replication timing for human chromosomes 11q and 21q: disease-related genes in timing-switch regions. Hum Mol Genet 11:13–21
45. Pacek M, Walter JC (2004) A requirement for MCM7 and Cdc45 in chromosome unwinding during eukaryotic DNA replication. Embo J 23:3667–3676
46. Musio A, Montagna C, Mariani T et al (2005) SMC1 involvement in fragile site expression. Hum Mol Genet 14:525–533
47. Saintigny Y, Delacote F, Vares G et al (2001) Characterization of homologous recombination induced by replication inhibition in mammalian cells. Embo J 20:3861–3870
48. Sancar A, Lindsey-Boltz LA, Unsal-Kacmaz K, Linn S (2004) Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. Annu Rev Biochem 73:39–85
49. Helmrich ABM, Tora L (2011) Collisions between replication and transcription complexes cause common fragile site instability at the longest human genes. Mol Cell 44:966–977
50. Jiang Y, Lucas I, Young DJ et al (2009) Common fragile sites are characterized by histone hypoacetylation. Hum Mol Genet 18:4501–4512
51. Vogelauer M, Rubbi L, Lucas I, Brewer BJ, Grunstein M (2002) Histone acetylation regulates the time of replication origin firing. Mol Cell 10:1223–1233

52. Barlow JH, Faryabi RB, Callen E et al (2013) Identification of early replicating fragile sites that contribute to genome instability. Cell 152:620–632
53. LeBeau MM, Rowley JD (1984) Heritable fragile sites in cancer. Nature 308:607–608
54. Yarbro JW (1992) Mechanism of action of hydroxyurea. Semin Oncol 19:1–10
55. Hills SA, Diffley JF (2014) DNA replication and oncogene-induced replicative stress. Curr Biol CB 24:R435–R444
56. Cerami E, Gao J, Dogrusoz U et al (2012) The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov 2:401–404
57. Gao J, Aksoy BA, Dogrusoz U et al (2013) Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal 6:pl1
58. Jiang Y, Hatzi K, Shaknovich R (2013) Mechanisms of epigenetic deregulation in lymphoid neoplasms. Blood 121:4271–4279
59. Abdel-Wahab O, Levine RL (2013) Mutations in epigenetic modifiers in the pathogenesis and therapy of acute myeloid leukemia. Blood 121:3563–3572
60. Berdasco M, Esteller M (2010) Aberrant epigenetic landscape in cancer: how cellular identity goes awry. Dev Cell 19:698–711
61. Arlt MF, Durkin SG, Ragland RL, Glover TW (2006) Common fragile sites as targets for chromosome rearrangements. DNA Repair 5:1126–1135
62. Iliopoulos D, Guler G, Han SY et al (2006) Roles of FHIT and WWOX fragile genes in cancer. Cancer Lett 232:27–36
63. Drusco A, Pekarsky Y, Costinean S et al (2011) Common fragile site tumor suppressor genes and corresponding mouse models of cancer. J Biomed Biotechnol 2011:984505
64. Saldivar JC, Bene J, Hosseini SA et al (2013) Characterization of the role of Fhit in suppression of DNA damage. Adv Biol Regul 53:77–85
65. Miller CT, Lin L, Casper AM et al (2006) Genomic amplification of MET with boundaries within fragile site FRA7G and upregulation of MET pathways in esophageal adenocarcinoma. Oncogene 25:409–418
66. Ciullo M, Debily MA, Rozier L et al (2002) Initiation of the breakage-fusion-bridge mechanism through common fragile site activation in human breast cancer cells: the model of PIP gene duplication from a break at FRA7I. Hum Mol Genet 11:2887–2894
67. Wilke CM, Guo SW, Hall BK et al (1994) Multicolor FISH mapping of YAC clones in 3p14 and identification of a YAC spanning both FRA3B and the t(3;8) associated with hereditary renal cell carcinoma. Genomics 22:319–326
68. Yamakawa K, Takahashi E, Murata M, Okui K, Yokoyama S, Nakamura Y (1992) Detailed mapping around the breakpoint of (3;8) translocation in familial renal cell carcinoma and FRA3B. Genomics 14:412–416
69. Chesi M, Bergsagel PL, Shonukan OO et al (1998) Frequent dysregulation of the c-maf proto-oncogene at 16q23 by translocation to an Ig locus in multiple myeloma. Blood 91:4457–4463
70. Nambiar M, Raghavan SC (2011) How does DNA break during chromosomal translocations? Nucleic Acids Res 39:5813–5825
71. Klein IA, Resch W, Jankovic M et al (2011) Translocation-capture sequencing reveals the extent and nature of chromosomal rearrangements in B lymphocytes. Cell 147:95–106
72. Rassool FV, McKeithan TW, Neilly ME, van Melle E, Espinosa R 3rd, Le Beau MM (1991) Preferential integration of marker DNA into the chromosomal fragile site at 3p14: an approach to cloning fragile sites. Proc Natl Acad Sci U S A 88:6657–6661
73. Wilke CM, Hall BK, Hoge A, Paradee W, Smith DI, Glover TW (1996) FRA3B extends over a broad region and contains a spontaneous HPV16 integration site: direct evidence for the coincidence of viral integration sites and fragile sites. Hum Mol Genet 5:187–195
74. Thorland EC, Myers SL, Gostout BS, Smith DI (2003) Common fragile sites are preferential targets for HPV16 integrations in cervical tumors. Oncogene 22:1225–1237
75. Bester AC, Roniger M, Oren YS et al (2011) Nucleotide deficiency promotes genomic instability in early stages of cancer development. Cell 145:435–446

76. Spardy N, Duensing A, Hoskins EE, Wells SI, Duensing S (2008) HPV-16 E7 reveals a link between DNA replication stress, fanconi anemia D2 protein, and alternative lengthening of telomere-associated promyelocytic leukemia bodies. Cancer Res 68:9954–9963
77. Ying S, Minocherhomji S, Chan KL et al (2013) MUS81 promotes common fragile site expression. Nat Cell Biol 15:1001–1007

Chapter 6
Copy Number Changes in Carcinomas: Applications

Henry Wood and Pamela Rabbitts

Contents

6.1 Introduction 96
6.2 Relationship Between Oncogenes and Chromosomal Amplifications 96
6.3 Chromosomal Deletions and Tumour Suppressor Genes 98
6.4 Identification of Functionally Important Cancer Genes 99
6.5 Copy Number Changes Associated with Disease Outcome 100
6.6 Genome-Wide Assessment of Copy Number Changes 101
References 101

Abstract The applications of information on copy number changes in cancer have been twofold. Recognizing that regions of copy number gain signalled the location of oncogenes and that, similarly, copy number loss signalled the location of tumour suppressor genes, has resulted in screening of the minimally defined regions for candidate genes involved in tumourigenesis. Once candidates emerged, other evidence of their role in tumours was sought, by functional assays for example, and a huge literature built up describing these gene classes. Even without knowledge of how the genes acted in the development of tumours, the second application has been to correlate the chromosomal abnormalities with various clinical parameters, again resulting in many thousands of publications, although to date the translation of laboratory observations into clinical practice is still not widespread.

Keywords Amplification • Deletion • Oncogene • Tumour suppressor gene • Clinical outcome

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6.1 Introduction

Early studies of tumour karyotypes used direct observation of chromosomes and many examples of abnormalities were observed. Two technical advances moved the field forward: firstly, the development of culture media to grow cancer cells in vitro and, secondly, the discovery that colchicine arrests cells in metaphase making their chromosomes visible using microscopy. When possible, cells growing in short-term cultures were passaged until they became established as immortalized cell-lines and these remain a valuable tool for molecular cell biology. When staining techniques were developed and each individual chromosome could be distinguished [1], tumour-specific chromosomal abnormalities could be enumerated. This enabled the construction of large catalogues such as the *Catalog of Chromosome Aberrations in Cancer* [2] now online but originally a hard-back book. Most of the catalog was devoted to haematological malignancies due to the relative ease of obtaining chromosome spreads from bone marrow or peripheral blood specimens. Solid tumours were often represented by samples from metastatic lesions or effusions and, thus, only typical of late stage disease.

The development of molecular genetics, made possible by DNA cloning, completely changed the approach to the study of copy number in tumours (see Chap. 2). It was no longer necessary to be able to observe chromosomes directly: instead, by measuring the relative proportions of DNA from different regions of the genome, chromosomal gain and loss could be inferred. This meant that it was possible to analyze relatively large numbers of primary tumour samples from patients although the extensive use of cell lines continued. Rather than whole chromosomes or chromosome arms, the better resolution of newer techniques allowed the emphasis to shift to focal regions since this provided the opportunity to identify which genes were involved. In the 1980s and 1990s, the focus was on understanding the biological consequences of gene loss and gain because it became clear that gene copy number gain was a mechanism of activating oncogenes and that gene copy number loss was a method of inactivating tumour suppressor genes. By the end of the twentieth century, conventional chemotherapy was recognized as failing to deliver the hoped for improvements in survival in the major common malignances: understanding the way in which these genes, collectively cancer genes, initiate and maintain the disease was seen as a new approach to identifying drug targets.

6.2 Relationship Between Oncogenes and Chromosomal Amplifications

The chromosomal abnormalities, homogeneously staining regions and double minutes, have been found exclusively in mammalian tumour cells, particularly in cell lines. Using Southern blotting and fluorescence in situ hybridization (FISH) (see Chap. 2), correlative evidence in mouse adrenal tumours suggested that these structures might be the location of amplified genes [3]. The same techniques were used

to make the link between a region of gene amplification and the location of a known oncogene. Since oncogenes, first found in avian retroviruses were known to have a cellular counterpart [4], a cell line, COLO320, with structural evidence of gene amplification, was screened for over-expression of 12 viral oncogenes: only the homolog of viral *myc* was overexpressed [5]. Neither the mechanisms of amplification of the homolog called cMYC nor the biological consequences of its over-expression were known at that time. However this study, by producing probes by molecular cloning for both filter and in situ hybridization, established a valuable approach to associate oncogenes with regions of gene amplification. To be successful, this trio of complementary methodologies worked best on cell-lines and, therefore, was most frequently applied to those tumours for which a large number of cell-lines had been derived. Lung tumours are a good example. In the 1980s, John Minna and Adi Gazdar had considerable success in developing the conditions required to establish lung tumours in culture and showed that members of the MYC family *MYC*, *MYCL*, and *MYCN* were frequency amplified and expressed [6]. Since not all tumour samples can be converted to cell lines, there was concern that the successful group represented the most aggressive diseases but parallel analyses of cell lines and their cognate primary tumours largely dispelled this concern [7].

Not all amplified regions have the benefit of encompassing a homolog of a viral oncogene to guide identification of the pivotal gene, but those amplicons that occur with high frequency have been the subject of intense scrutiny. A good example of this is the amplified regions on 3q. This is very common in all squamous cell carcinomas sometimes occurring as an extra copy of the chromosome arm, as in cervical tumours [8], but also involving minimal regions such as the focal amplifications seen at 3q26 in squamous cell lung cancers [9]. Even then, the region encompasses a large number of genes: several methods have been used to determine the key gene(s) within the amplicon. Often this starts with an educated guess evolving from a knowledge of the characteristics of genes within the amplified region: in the 3q amplicon *TP63*, *P3CA* and *SOX2* have all been favoured [10]. In a few oesophageal tumours, copy number analysis has pinpointed *SOX2* as the only amplified gene in the amplicon. Functional analysis confirmed its role in tumour proliferation when co-transfection with *FOXE1* or *FGFR2* transformed an immortalized (but non-tumourigenic) bronchial epithelial cell-line [11]. In another study *SOX2* and another 3q26 gene, *PPKC1* were shown to cooperate to activate hedgehog signalling in a cell model of squamous cell lung cancer [12]. Taking a computational approach to identify co-operating genes within the amplicon identified a further three genes: *SENP2*, *DCUN1D1* and *DVL3* [13]. Confusingly, increased expression has been associated with increased survival in some lung cancer patients [14] and decreased survival in patients with cervical cancer [15].

The narrative of this research, designed to identify the pivotal genes in this very important amplicon, illustrates a current problem. Although the gene order on chromosomes, their copy number and transcription levels are now well documented by high through-put sequencing and the use of expression microarrays, functional assays to confirm the key gene(s) in an amplicon have not kept pace with structural analysis.

6.3 Chromosomal Deletions and Tumour Suppressor Genes

Investigators using classical cytogenetic techniques were able to identify deletions but it was the application of a genetic and molecular genetic approach to a childhood tumour, retinoblastoma [16] that captured their importance for the development of cancer and identified a new class of genes, later called tumour suppressor genes, characterized by the requirement for inactivation of both alleles to elicit a tumourigenic effect – the 'two hit mechanism' [17]. Just as viral oncogenes were of value to pinpoint oncogenes involved in human tumours, for tumour suppressor genes (TSGs), inherited cancer syndromes provided a useful route to identify their chromosomal location [18]. Genetic linkage studies were used first to define the chromosomal locus followed by molecular genetic approaches, such as loss of heterozygosity analysis, LOH (see Chap. 2) to define the region further and identify genes that could be examined for mutations by sequence analysis.

A number of TSGs have been identified using this approach such as *APC* [19], *BRCA1* [20] and *BRCA2* [21]. In some situations, the role of the deletions is to delineate candidate genes; in others, finding that a gene already associated with cancer within a deletion can be a validation of its authenticity. This is true for TP53 which was first isolated as a host protein binding to a tumour viral protein (SV40 large T), but gained its tumour suppressor gene status when it was shown to reside in a frequently deleted region on chromosome 17 in colon tumours [22]. In the same study, the gene was shown to be mutated by sequence analysis. Subsequently, *TP53* has been found to be involved in at least 50 % of human cancers [23], and is the subject of tens of thousands of research articles, yet despite being frequently mutated, it has not yet found its way into routine clinical practice either as a disease marker or a drug target [24].

The success in using deletions to pinpoint TSGs was next applied to cancers with no obvious inherited predisposition, since cytogenetic analysis of chromosomes spreads showed evidence of frequent deletions in solid tumours [1]. This was followed by LOH analysis of samples from much larger patient series hoping to define a minimally deleted region to reduce the number of genes that required scrutiny for the presence of mutations. This was a daunting task [24] especially before the human genome was sequenced and the number and order of genes on chromosomes was known. However, both alleles are inactivated by a homozygous deletion within the region of interest in some tumours, thereby limiting the number of genes to be examined, as the deletion has to be compatible with cell viability. Such deletions were used successfully in the identification of a number of TSG, *p16*/*CDKN2A* [25] and *PTEN* [26] being notable examples. But not all homozygous deletions harbour *bona fide* TSG [27]. Studies on chromosome 3 exemplify this. Deletions of or within the short arm of chromosome 3 are very common in a range of malignant tumours, especially those of the squamous subtype, and occur very early in the development of these tumours and are even occasionally detected in apparently normal epithelium cells (see Chap. 5). Many studies have scrutinized the genes residing in homozygous deletions in 3p without

identifying genes showing frequent mutation [28]. One possible explanation is that although deletion is responsible for the loss of one allele the remaining allele is inactivated by an epigenetic mechanism such as methylation [29]. Although candidate TSGs on 3p were identified, such as *RASSF1* and *FHIT*, and partly validated, their inactivation in mouse models did not produce robust evidence of their independent tumour suppressor function. Furthermore it is becoming clear that, without the benefit of homozygous deletions, LOH is a clumsy tool for positional cloning strategies [24]. Nonetheless a more recent evaluation by the originator of the "two hit hypothesis", Alfred Knudson, concedes that TSGs may have a role in tumourigenesis through their partial inactivation, and the concept of haploin sufficiency has now been validated for a number of TSGs [30]. A recent example of what this might mean is a study in renal cell carcinoma which showed that genes involved in LOH adjacent to the *VHL* gene, a TSG with a known role in this cancer, were down-regulated, resulting in a network metabolism signature unique to this cancer [31]. Thus, the "one gene at a time" approach that worked so well in the early phase of TSG discovery may be too simplistic and cooperation between genes may be involved in somatically arising tumours [32].

6.4 Identification of Functionally Important Cancer Genes

The identification of consistent copy number changes, amplifications and deletions, can provide strong circumstantial evidence for the involvement of the delineated genes in tumourigenesis. If genes within the candidate regions are frequently mutated in a tumour-specific manner, this greatly increases the conviction that the gene is directly involved in tumour development. Even in this situation, and certainly when candidates have no recurring mutations, functional assays as mentioned above, are needed to confirm the gene's status, and also to understand the way in which a mutant protein has a tumourigenic effect. Assays for oncogenes have depended on introducing the suspected genes as a cDNA into untransformed cells and scoring for a tumour-related phenotype, usually involving increased proliferation [33]. Conversely, tumour-suppressing potential is assessed by introducing the suspected gene into tumour cell lines and observing a decrease in tumour-related features, such as migration or colony formation [34]. Assays involving tumour formation in nude mice have also been used [35] although, more recently, genetically engineered mice have been the system of choice to recapitulate gene expression in human tumours more closely as described in Chap. 20 in this book. However, traditionally this has been a time-consuming and expensive method. More recently, using the increased data now available for both genomes and transcriptomes, computational methods to identify pathways or networks and expose driver genes have become prevalent [36, 37]. Additionally, biological screens, such as RNAi, are being developed to replace the single gene approach [38]. A recent review of all these methods is provided by Eifert and Powers [39].

6.5 Copy Number Changes Associated with Disease Outcome

Naturally with so many chromosomal regions and interesting genes associated with cancer, the question that is frequently asked is 'do the genomic and genetic abnormalities have any clinical significance?' For these translational studies, a knowledge of the gene function is not required; in fact the correlation of a genetic abnormality and a clinically-related phenotype can be another way in which evidence is accumulated to support the importance of a particular gene. Clinical utility ranges across diagnosis, prognosis and prediction of treatment response, including efficacy and toxicity.

An early success was the association of the *MYCN* gene and neuroblastoma. Following on from the discovery that the *MYC* gene is localized to an amplified region in lung tumours [40], other tumour types with known amplifications were tested with probes to *MYC*. In this way, a gene homologous to *MYC* was found to be amplified in neuroblastoma and called *MYCN* [41]. It was of particular interest because the degree to which *MYCN* was amplified was closely associated with the disease stage, demonstrating its value as a prognostic marker [42].

The greatest success in translating laboratory discoveries into the clinic has been obtained for breast cancer. An early observation was that the *ERBB2* gene, more usually now called *HER2*/*neu* or just *HER2*, was amplified and over-expressed in breast cancer, and that this indicated a poor prognosis [43]. The development of an antibody to the HER2 protein, a receptor on the cell surface, that was shown to be effective in the treatment of HER2 "positive" breast cancer [44, 45] meant that it was essential to develop robust laboratory tests to identify patients who would benefit from HER2-targeted therapies [46]. These tests rely on FISH to detect gene amplification or immunohistochemistry to detect increased levels of the HER2 protein. As such, these tests are only semi-quantitative and subjective and rely on experienced professionals for their interpretation. There is clearly a place for a test based on direct assessment of the patients' tumour DNA and high through-put sequencing should provide that, although issues of normal cell contamination and inter-tumour heterogeneity will have their own drawbacks.

Although many thousands of studies of both genes and chromosomal regions have been linked to cancer phenotypes [47, 48] described in a database [49], only a very small number have been developed for use in the clinic, such as *HER2* and *EGFR*. There are a number of reasons for this. With regard to prognosis, recurrence and survival, often the information does not directly impact on clinical management because the number of treatment options is limited and there are other confounding factors involved in their selection. A further complication that is currently receiving attention, is the effect of intra-tumour heterogeneity on the distribution of markers and targets [50]. This heterogeneity could result in biopsies failing to reflect the molecular composition of the whole tumour with obvious consequences for clinical management. It has also been appreciated that the conceptual and statistical framework applied to clinical trials needs to be developed for biomarker studies [51]. This will be particularly important for those biomarkers developed for the prediction of

treatment response, including toxicity, as the biomarker would have immediate clinical application [52].

6.6 Genome-Wide Assessment of Copy Number Changes

When gene expression data are used to inform clinical outcomes, rather than single genes, groups of genes are assessed in a single test [53]. This approach may prove useful for copy number data. Although genome-wide copy number evaluation using comparative genomic hybridization (CGH) has been used extensively to provide copy number read-out across the genome, analysis has not usually been of whole genomes, but rather to pinpoint regions of particular interest (see Chap. 2). However, it is possible to use DNA copy number data obtained using microarrays to define patterns of gain and loss within the genome that have distinct relationships with outcomes. Hicks et al. [54] showed that when the whole genomes of breast cancers were defined by "the number and proximity of genomic alterations" they could be segregated into groups with different overall survival. Consideration of the whole genome may be less vulnerable to inter-tumour heterogeneity since many parameters are being assessed simultaneously. Single gene tests have the problem that they might implicate a candidate driver which in reality is only amplified or deleted in a fraction of the tumour mass. They have the additional problem that the driver for any particular tumour might not be the gene being tested. With whole genome measurements, the real drivers will be more likely to be present at an early stage in the disease, so are detected more frequently. If whole genomes are used to identify candidate genes, then computational methods need to be employed to filter all the potential drivers. These whole genome signatures are usually less reliant on the copy number in any one gene, instead measuring the cumulative effects of multiple regions of the genome, or the entire genome. Although microarray analysis has been the most common method for whole genome copy number measurement in recent years, the advent of next-generation sequencing has seen this monopoly eroded. Campbell et al. first described copy number measurement using next-generation sequencing in 2008 [55]. Since then, it has been shown to be possible to use very low coverage data to produce a similar read-out to the microarray method using diagnostic material at low cost [56] making it suitable for clinical use.

References

1. Caspersson T, Zech L, Johansson C (1970) Differential binding of alkylating fluorochromes in human chromosomes. Exp Cell Res 60:315–319
2. Mitelman (ed) (1983) Catalog of chromosome aberrations in cancer. Wiley-Liss
3. George D, Powers V (1982) Amplified DNA sequences in Y1 mouse adrenal tumor cells: association with double minutes and localization to a homogeneously staining chromosomal region. Proc Natl Acad Sci U S A 79:1597–1601

4. Bishop J (1983) Cellular oncogenes and retroviruses. Annu Rev Biochem 52:301–354
5. Alitalo K, Schwab M, Lin C, Varmus H, Bishop J (1983) Homogeneously staining chromosomal regions contain amplified copies of an abundantly expressed cellular oncogene (c-myc) in malignant neuroendocrine cells from a human colon carcinoma. Proc Natl Acad Sci U S A 80:1707–1711
6. Johnson B, Brennan J, Ihde D, Gazdar A (1992) myc family DNA amplification in tumors and tumor cell lines from patients with small-cell lung cancer. J Natl Cancer Inst Monogr 13:39–43
7. Johnson B, Makuch R, Simmons A, Gazdar A, Burch D et al (1988) myc family DNA amplification in small cell lung cancer patients' tumors and corresponding cell lines. Cancer Res 48:5163–5166
8. Ma Y, Wei S, Lin Y, Lung J, Chang T et al (2000) PIK3CA as an oncogene in cervical cancer. Oncogene 19:2739–2744
9. Qian J, Massion P (2008) Role of chromosome 3q amplification in lung cancer. J Thorac Oncol 3:212–215
10. McCaughan F, Pole J, Bankier A, Konfortov B, Carroll B et al (2010) Progressive 3q amplification consistently targets SOX2 in preinvasive squamous lung cancer. Am J Respir Crit Care Med 182:83–91
11. Bass A, Watanabe H, Mermel C, Yu S, Perner S et al (2009) SOX2 is an amplified lineage-survival oncogene in lung and esophageal squamous cell carcinomas. Nat Genet 41:1238–1242
12. Justilien V, Walsh M, Ali S, Thompson E, Murray N et al (2014) The PRKCI and SOX2 oncogenes are coamplified and cooperate to activate Hedgehog signaling in lung squamous cell carcinoma. Cancer Cell 10:139–151
13. Wang J, Qian J, Hoeksema M, Zou Y, Espinosa A et al (2013) Integrative genomics analysis identifies candidate drivers at 3q26-29 amplicon in squamous cell carcinoma of the lung. Clin Cancer Res 19:5580–5590
14. Toschi L, Finocchiaro G, Nguyen T, Skokan M, Giordano L et al (2014) Increased SOX2 gene copy number is associated with FGFR1 and PIK3CA gene gain in non-small cell lung cancer and predicts improved survival in early stage disease. PLoS ONE 9:e95303
15. Shen L, Huang X, Xie X, Su J, Yuan J et al (2014) High expression of SOX2 and OCT4 indicates radiation resistance and an independent negative prognosis in cervical squamous cell carcinoma. J Histochem Cytochem 62:499–509
16. Hansen M, Cavenee W (1988) Retinoblastoma and the progression of tumor genetics. Trends Genet 4:125–128
17. Knudson AG Jr (1971) Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci U S A 68:820–823
18. White R (1992) Inherited cancer genes. Curr Opin Genet Dev 2:53–57
19. Levy D, Smith K, Beazer-Barclay Y, Hamilton S, Vogelstein et al (1994) Inactivation of both APC alleles in human and mouse tumors. Cancer Res 54:5953–5958
20. Smith S, Easton D, Evans D, Ponder B (1992) Allele losses in the region 17q12-21 in familial breast and ovarian cancer involve the wild-type chromosome. Nat Genet 2:128–131
21. Gudmundsson J, Johannesdottir G, Bergthorsson J, Arason A, Ingvarsson S et al (1995) Different tumor types from BRCA2 carriers show wild-type chromosome deletions on 13q12-q13. Cancer Res 55:4830–3832
22. Baker S, Fearon E, Nigro J, Hamilton S, Preisinger A et al (1989) Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science 244:217–221
23. Hollstein M, Sidransky D, Vogelstein B, Harris C (1991) p53 mutations in human cancers. Science 253:49–53
24. Devilee P, Cleton-Jansen A, Cornelisse C (2001) Ever since Knudson. Trends Genet 17:569–573
25. Kamb A, Gruis N, Weaver-Feldhaus J, Liu Q, Harshman K et al (1994) A cell cycle regulator potentially involved in genesis of many tumor types. Science 264:436–440

26. Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI et al (1997) PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science 275:1943–1947
27. Cox C, Bignell G, Greenman C, Stabenau A, Warren W et al (2005) A survey of homozygous deletions in human cancer genomes. Proc Natl Acad Sci U S A 102:4542–4547
28. Kok K, Naylor S, Buys C (1997) Deletions of the short arm of chromosome 3 in solid tumors and the search for suppressor genes. Adv Cancer Res 71:27–92
29. Burbee D, Forgacs E, Zöchbauer-Müller S, Shivakumar L, Fong K et al (2011) Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. J Natl Cancer Inst 93:691–699
30. Berger A, Knudson A, Pandolfi P (2011) A continuum model for tumor suppression. Nature 476:163–169
31. Gatto F, Nookaew I, Nielsen J (2014) Chromosome 3p loss of heterozygosity is associated with a unique metabolic network in clear cell renal carcinoma. Proc Natl Acad Sci U S A 111:E866–E875
32. Watanabe H, Ma Q, Peng S, Adelmant G, Swain D et al (2014) SOX2 and p63 colocalize at genetic loci in squamous cell carcinomas. J Clin Invest 124:1636–1645
33. Booden MA, Ulka AS, Der CJ (2006) Cellular assays of oncogene transformation. In: Cellis JE (ed) Cell biology a laboratory handbook, published by Elsevier, pp 345–352
34. Brummelkamp T, Bernards R, Agami R (2002) A system for stable expression of short interfering RNAs in mammalian cells. Science 296:550–553
35. Engel AM, Schou G (2006) Assays of tumorigencity in nude mice. In: Cellis JE (ed) Cell biology a laboratory handbook, published by Elsevier, pp 353–357
36. Akavia U, Litvin O, Kim J, Sanchez-Garcia F, Kotliar D et al (2010) An integrated approach to uncover drivers of cancer. Cell 143:1005–1017
37. Santarius T, Shipley J, Brewer D, Stratton MR, Cooper C (2010) A census of amplified and overexpressed human cancer genes. Nat Rev Cancer 10:59–64
38. Sawey E, Chanrion M, Cai C, Wu G, Zhang J et al (2011) Identification of a therapeutic strategy targeting amplified FGF19 in liver cancer by Oncogenomic screening. Cancer Cell 19:347–358
39. Eifert C, Powers R (2012) From cancer genomes to oncogenic drivers, tumour dependencies and therapeutic targets. Nat Rev Cancer 12:572–578
40. Soucek L, Evan G (2010) The ups and downs of Myc biology. Curr Opin Genet Dev 20:91–95
41. Schwab M, Alitalo K, Klempnauer KH, Varmus HE, Bishop J et al (1983) Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. Nature 305:245–248
42. Brodeur G, Seeger R, Schwab M, Varmus H, Bishop J (1984) Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. Science 224:1121–1124
43. Slamon D, Clark G, Wong S, Levin W, Ullrich A et al (1987) Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235:177–182
44. Cobleigh M, Vogel C, Tripathy D, Robert N, Scholl S et al (1999) Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. J Clin Oncol 17:2639–2648
45. Verma S, Miles D, Gianni L, Krop IE, Welslau M et al (2012) Trastuzumab emtansine for HER2-positive advanced breast cancer. N Engl J Med 367:1783–1791
46. Rakha E, Ellis O (2014) Breast cancer: updated guideline recommendations for HER2 testing. Nat Rev Clin Oncol 11:8–9
47. Myllykangas S, Himberg J, Böhling T, Nagy B, Hollmén J et al (2006) DNA copy number amplification profiling of human neoplasms. Oncogene 25:7324–7332

48. Myllykangas S, Böhling T, Knuutila S (2007) Specificity, selection and significance of gene amplifications in cancer. Semin Cancer Biol 17:42–55
49. Scheinin I, Myllykangas S, Borze I, Böhling T, Knuutila S et al (2008) CanGEM: mining gene copy number changes in cancer. Nucleic Acids Res 36:D830–D835
50. Crockford A, Jamal-Hanjani M, Hicks J, Swanton C (2014) Implications of intratumour heterogeneity for treatment stratification. Pathology 232:264–273
51. Poste G (2012) Biospecimens, biomarkers, and burgeoning data: the imperative for more rigorous research standards. Trends Mol Med 18:717–722
52. Mehta S, Shelling A, Muthukaruppan A, Lasham A, Blenkiron C et al (2010) Predictive and prognostic molecular markers for cancer medicine. Ther Adv Med Oncol 2:2125–2148
53. Chen HY, Yu SL, Chen CH, Chang GC, Chen CY et al (2007) A five-gene signature and clinical outcome in non-small-cell lung cancer. N Engl J Med 356:11–20
54. Hicks J, Krasnitz A, Lakshmi B, Navin NE, Riggs M et al (2006) Novel patterns of genome rearrangement and their association with survival in breast cancer. Genome Res 16:1465–1479
55. Campbell P, Stephens P, Pleasance E, O'Meara S, Li H et al (2008) Identification of somatically acquired rearrangements in cancer using genome-wide massively parallel paired-end sequencing. Nat Genet 40:722–729
56. Wood H, Belvedere O, Conway C, Daly C, Chalkley R et al (2010) Using next generation sequencing for high resolution multiplex analysis of copy number variation from nanogram quantities of DNA from formalin fixed paraffin embedded specimens. Nucl Acid Res 38:e151

Part III
Leukaemia/Lymphoma

Chapter 7
Chronic Myeloid Leukaemia

Debora A. Casolari and Junia V. Melo

Contents

7.1 Introduction 108
7.2 CML Characteristics and Disease Phases 108
7.3 Molecular Pathogenesis 109
7.3.1 The *BCR-ABL1* Gene 109
7.3.2 The BCR-ABL1 Protein 111
7.3.3 Signalling and Disease 113
7.3.3.1 Proliferation and Survival 113
7.3.3.2 Progression to Blast Crisis 115
7.4 Targeted Therapy 117
7.4.1 First Generation TKI: Imatinib 117
7.4.1.1 Resistance to IM 118
7.4.2 Second and Third Generation TKIs 120
7.4.2.1 Dasatinib 120
7.4.2.2 Nilotinib 120
7.4.2.3 Bosutinib 121
7.4.2.4 Ponatinib 121
7.4.2.5 Rebastinib 121
7.5 LSC as a Therapeutic Target in CML 121
7.6 Conclusion 126
References 126

Abstract Chronic myeloid leukaemia (CML) was the first leukaemia associated with a unique genetic abnormality, the Philadelphia chromosome. This results from a reciprocal translocation between chromosomes 9 and 22, which generates the *BCR-ABL1* fusion gene encoding a constitutively active tyrosine kinase. The complex intracellular signalling initiated by BCR-ABL1 is responsible for disease development, and targeted tyrosine kinase inhibitors have been the most successful therapeutic advance in CML. In this chapter, we review the implications of

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BCR-ABL1 signalling in CML, how this knowledge revolutionized CML treatment, and discuss approaches to further improving therapeutic response by the targeting of leukaemic stem cells.

Keywords Chronic myeloid leukaemia • BCR-ABL1 • Tyrosine kinase inhibitor • Blast crisis • Leukaemic stem cell

7.1 Introduction

Chronic myeloid leukaemia (CML) was probably the first form of leukaemia to be independently recognized in 1845 by John H. Bennett, in Scotland, and Rudolf Virchow, in Germany [1]. The discovery of the Philadelphia (Ph) chromosome, in 1960 [2] was the first consistent chromosomal abnormality associated with a specific type of leukaemia, and was not only a breakthrough in cancer biology but also the first important clue to CML pathogenesis. In 1973, Rowley identified the Ph chromosome as a shortened chromosome 22 (22q-), result of a reciprocal t(9;22)(q34.1;q11.2) translocation [3]. In the next decade, the Ph chromosome was shown to carry a unique fusion gene, *BCR-ABL1* [4], the deregulated BCR-ABL1 tyrosine kinase activity was defined as the pathogenetic principle of CML [5], and the first animal models were developed [6]. Ultimately, this knowledge provided the basis for the design of a targeted therapy for CML with the development of ABL1 specific tyrosine kinase inhibitors (TKIs), which selectively inhibit the growth of *BCR-ABL1* positive cells *in vitro* and *in vivo* [7–9].

7.2 CML Characteristics and Disease Phases

CML is a clonal myeloproliferative disease originating in a single haematopoietic stem cell (HSC). It represents 15–20 % of the leukaemias in adults and has a relatively low incidence (1–1.5 new cases per 100,000 people per year). However, its prevalence is on the rise due to the significant improvement in its treatment over the past 15 years [10]. In the Western countries, the median age of patients at diagnosis is 55–65 years old, whereas it is significantly lower, averaging 38–41 years, in Asia, Africa, Southern/Eastern Europe and Latin America [11]. The disease affects both sexes, with a slight male preponderance (male:female ratio of 1.3:1).

The only known predisposing factor to CML is high-dose ionizing radiation, as best demonstrated by studies of survivors of the Hiroshima and Nagasaki atomic bomb explosions [12]. Apart from a borderline increased risk of CML in first-degree relatives of patients with myeloproliferative disorders [13], there is no evidence of an inherited disposition or association with chemical exposure.

In its natural history, CML is a tri-phasic disease, predominantly presenting in a chronic phase (CP) averaging around 3–7 years. In most cases of CP CML, the neoplastic expansion involves a leukaemic clone that differentiates into mature granulocytes which function normally, despite being derived from malignant progenitors. The 'indolent' phenotype of CP means that some patients are asymptomatic, and the diagnosis is frequently an incidental finding; however, the majority typically present with mild symptoms of fatigue, weight loss and sweats [14, 15]. CP progresses to either the transitional accelerated phase (AP) or transforms directly into blast crisis (BC). When present, the AP precedes BC by 2–15 months [16]. Transformation to BC is characterized by the presence, in the peripheral blood or bone marrow (BM), of 20 % or more blasts, which can be of myeloid (approximately 70 % of cases) or lymphoid (30 %) origin [17]. BC is clinically indistinguishable from acute leukaemia and can present leukocytosis, cytopenia, hepatosplenomegaly, enlarged lymph nodes, and marked refractoriness to treatment which results in a dismal clinical outcome, with a historical median survival of no more than 3–6 months [18]. Even with the advent of TKIs, response of BC to this type of therapy is minimal, and median survival is still only 9 months [19, 20].

Until the emergency of TKIs, the only curative treatment for CML was HSC transplantation, but this was restricted to a minority of patients, due to age restrictions and the need for a histocompatible donor. The prognosis has now substantially improved for most CML patients who respond well to TKIs, a proportion of whom are able to survive indefinitely without evidence of disease.

7.3 Molecular Pathogenesis

7.3.1 The BCR-ABL1 Gene

The t(9;22)(q34.1;q11.2) reciprocal translocation gives rise to two pathognomonic fusion genes, *BCR-ABL1* on the der(22) (Ph) chromosome, and *ABL1-BCR*, on the der(9) (Fig. 7.1). Although the latter is transcribed, there is no evidence that it has functional relevance to the disease [21]. Thus, it is the translation of the *BCR-ABL1* gene into an abnormal fusion protein that is responsible for the leukaemic process.

The breakpoints within *ABL1* at 9q34.1 can occur anywhere over a large (>300 kb) area at its 5′ end, either upstream of exon 1b, downstream of exon 1a or, more frequently, between the two [22]. Regardless of the exact location of the breakpoint, splicing of the transcript yields an mRNA molecule where *BCR* is fused to *ABL1* exon a2 (Fig. 7.2).

In contrast to *ABL1*, breakpoints within *BCR* localize to one of three breakpoint cluster regions (bcr). In most CML cases and in about one third of Ph + acute lymphoblastic leukaemias (ALL), the break occurs within *BCR* exons 12–16 (previously exons b1–b5), defined as the major bcr (M-bcr) [22]. Due to alternative splicing, the mRNA usually contains the *BCR-ABL1* junctions e13a2 or e14a2

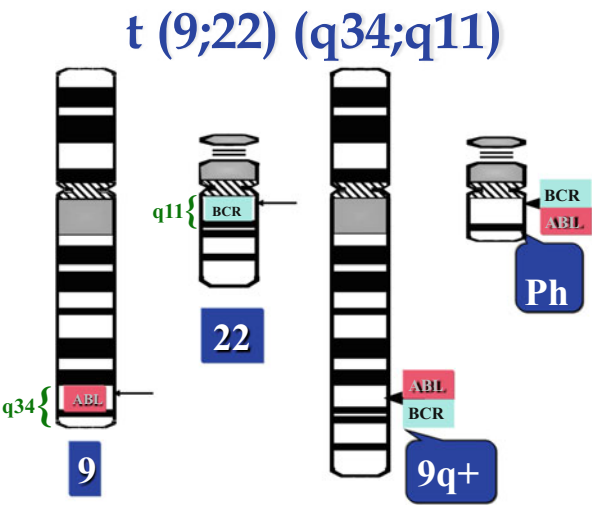

Fig. 7.1 Schematic representation of the t(9;22)(q34.1;q11.2) chromosomal translocation, the cytogenetic hallmark of chronic myeloid leukaemia. Breaks within the ABL1 and BCR genes on chromosomes 9 and 22, respectively, followed by recombination of the broken DNA ends, give origin to two derivative chromosomes, the der(22) or Philadelphia (Ph) and the der(9). The BCR-ABL1 gene, which is the pathogenetic product of this translocation, is housed in the Ph chromosome

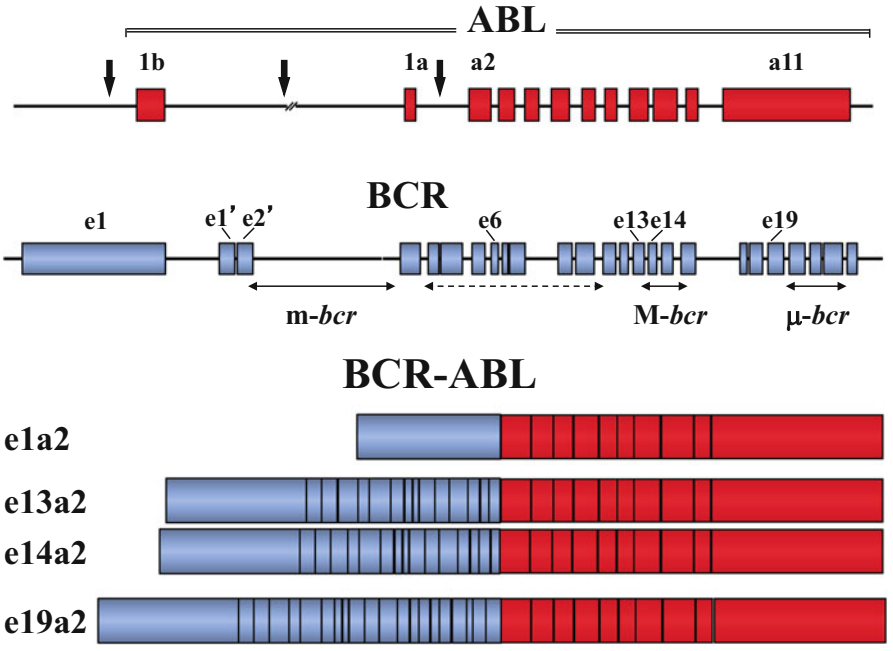

Fig. 7.2 Schematic representation of the ABL1 and the BCR genes disrupted in the t(9;22)(q34.1;q11.2). Exons are represented by *boxes* and introns by connecting *horizontal lines*. Breakpoint regions in ABL1 are illustrated as *vertical arrows*, and in BCR by the three double-headed *horizontal arrows*. The lower half of the figure shows the structure of the various BCR-ABL1 mRNA transcripts which are formed in accordance with the position of the breakpoint in BCR. Breaks in m-*bcr* give origin to BCR-ABL1 mRNA molecules with an e1a2 junction. The breaks in M-*bcr* occur either between exons e13 and e14 or between e14 and e15, generating fusion transcripts with a e13a2 or a e14a2 junction, respectively. Breakpoints in μ-*bcr*, the most 3′ cluster region, result in BCR-ABL1 transcripts with an e19a2 junction

(originally b2a2 or b3a2) and is translated into a 210 kDa protein ($P210^{BCR-ABL}$). The majority of Ph + ALL and very rare cases of CML, characterized by prominent monocytosis [23, 24], have breakpoints further upstream between exons e2′ and e2, termed the minor bcr (m-*bcr*). The resulting e1a2 mRNA is translated into a 190 kDa protein ($P190^{BCR-ABL}$). A third bcr (μ-*bcr*) is located downstream of exon 19, giving rise to a 230 kDa fusion protein ($P230^{BCR-ABL}$), which is sometimes associated with an uncommon neutrophilic variant of CML [25, 26]. Although all three major BCR-ABL1 fusion proteins induce a CML-like disease in mice, they differ in their ability to induce lymphoid leukaemia [27].

The mechanism by which the Ph chromosome is first formed and the time required for overt disease to appear are unknown. *BCR-ABL1* fusion transcripts can be induced in haematopoietic cells by exposure to ionizing radiation *in vitro* [28]; such induced translocations may not be random events but may depend on the cellular background and the particular genes involved. Translocations between *BCR* and *ABL1* may be favoured by their relative proximity during the interphase of cycling haematopoietic cells [29]. Furthermore, a 76 kb 'duplicon' near *ABL1* and *BCR* has been implicated in the translocation, but this mechanism is purely speculative [30].

The *BCR-ABL1* gene is expressed in all CML patients, but the reciprocal *ABL1-BCR* gene on the der(9) occurs in only 70 % of cases [21]. Approximately 20 % of CML patients have deletions on the der(9) and have significantly shorter survival than those lacking the deletions [31, 32]. Notably, absence of the *ABL1-BCR* gene, which is always included in the deleted region, does not by itself have the same ominous prognostic implication [33]. Similarly, no prognostic relevance of the der(9) deletions was observed on patients treated with TKIs [34, 35].

The idea that CML may result from a multi-step process was first broached over 30 years ago [36] but there is little evidence of additional abnormalities that precede the t(9;22) translocation. Even so, the presence of *BCR-ABL1* in any haematopoietic cell is not in itself sufficient to cause leukaemia, since *BCR-ABL1* is detectable at low frequency in the blood of many normal individuals [37, 38]. Thus the generation of a *correct BCR-ABL1* in a *multipotent HSC*, possibly under reduced immunological surveillance, is necessary to initiate the clonal expansion that leads to CML. This hypothesis is supported by the production of a CML-like disease in mice transplanted with *BCR-ABL1*-positive stem cells [6, 39, 40]. However, once established, the 'tempo' or aggressiveness of the CP disease varies in different patients and must be influenced by other factors.

7.3.2 The BCR-ABL1 Protein

The BCR-ABL1 oncoprotein includes several important domains of its parental BCR and ABL1 normal counterparts, which endow it of specific biological properties (Fig. 7.3).

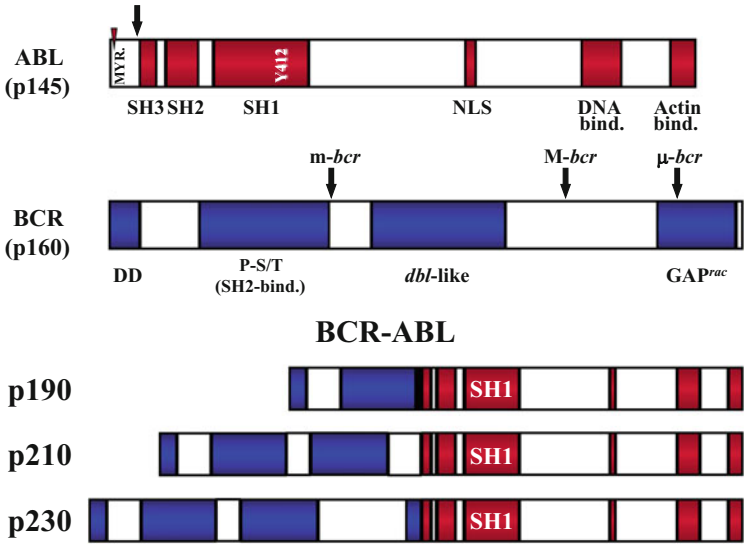

Fig. 7.3 Schematic representation of the normal ABL1 (p145), the normal BCR (p160) and the leukaemia-associated BCR-ABL1 fusion proteins. Note that the variation between the three forms of BCR-ABL1 proteins is due to the different contributions of BCR rather than of ABL1 sequences to the hybrid product. The arrows in BCR indicate the sites of protein fusion arising from m-*bcr* ($p190^{BCR\text{-}ABL}$), M-*bcr* ($p210^{BCR\text{-}ABL}$) and μ-*bcr* ($p230^{BCR\text{-}ABL}$) breakpoints. Some special features and regions of these proteins are shown: In the ABL1 protein these are the myristoylation (MYR) site present in the human type 1b protein, the regulatory *src*-homology (SH) regions SH3 and SH2, the SH1 (kinase domain) with its principal site of autophosphorylation (Y412), the nuclear localisation signal (NLS), the DNA- and the actin-binding domains. In the BCR protein these are the dimerization domain (DD), the phospho-serine/threonine (P-S/T)-rich SH2-binding domain, the *dbl*-like and the GAP^{rac} domains

In ABL1, they include the SRC-homology SH1, SH2 and SH3, a nuclear localisation signal, DNA and actin-binding domains, and in BCR a coiled-coil motif contained in amino acids 1–63 [41], the tyrosine at position 177 [42] and phospho-serine/threonine rich sequences between amino acids 192–242 and 298–413 [43]. The most important feature for its leukaemogenic potential resides in the fact that the tyrosine kinase of the ABL1 protein is constitutively activated by the juxtaposition of BCR. The BCR dimerization domains connect two BCR-ABL1 molecules which then phosphorylate their respective partners on tyrosine residues in the kinase activation loops [41]. The consequent increase of phosphotyrosine residues on BCR-ABL1 itself creates binding sites for the SH2 domains of other proteins. A host of substrates can be tyrosine phosphorylated by BCR-ABL1, the net result of which is deregulated cellular proliferation, decreased adherence of leukaemia cells to the BM stroma, reduced response to apoptotic stimuli, increased genomic instability and increased capacity for self-renewal [44, 45].

Tyrosine phosphatases counterbalance and regulate the effects of tyrosine kinases under physiological conditions. Two tyrosine phosphatases, SYP and PTPN1, have

been shown to form complexes with BCR-ABL1, and both appear to dephosphorylate BCR-ABL1 [46, 47]. On the other hand, BCR-ABL1 protects itself from the protein tyrosine phosphatase 1 (PTPN6/SHP1), which can dephosphorylate BCR-ABL1 and induce its proteasomal degradation, by inhibiting the PTPN6/SHP1 activator PP2A [48].

7.3.3 Signalling and Disease

7.3.3.1 Proliferation and Survival

BCR-ABL1 shifts the balance towards inhibition of apoptosis while simultaneously providing a proliferative stimulus through multiple signals. These are frequently difficult to separate but mostly involve PI3K/AKT1, JAK/STAT, RAS/RAF/MEK/ERK and MYC pathways (Fig. 7.4).

Once the adapter molecule GRB2 binds to P-Tyr177 on BCR-ABL1, it recruits SOS and constitutively activates RAS, which, in turn, activates MAPK3/ERK1 and MAPK1/ERK2 [42, 49]. Two other adapter molecules, SHC1 and CRKL, can also activate RAS after binding to BCR-ABL1 [50, 51]. Ultimately, activated MAPKs indirectly induce gene transcription and cell proliferation [49, 52].

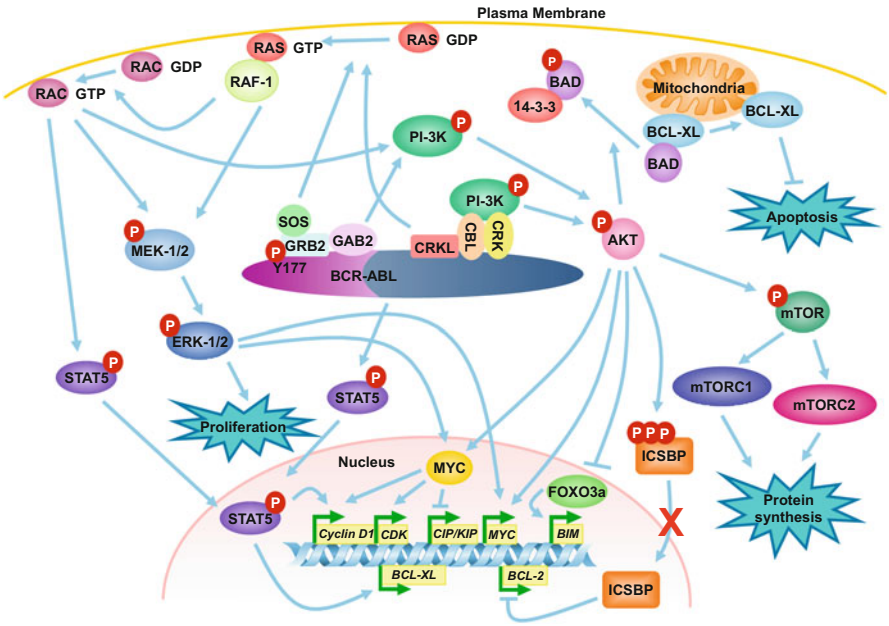

Fig. 7.4 Some of the major signalling pathways directly or indirectly regulated by BCR-ABL1 (see text for detailed descriptions)

Signalling from RAS can be relayed via RAC GTPases [53] to activate MAPK8/JNK, which is required for BCR-ABL1 malignant transformation [54]. Accordingly, downregulation of the JNK pathway negative regulator JUNB, by promoter hypermethylation, has been described in CML primary cells [55]. RAC GTPases themselves play an important role in BCR-ABL1 leukaemogenesis, activating STAT5, PI3K and MAPKs pathways [56]. Moreover, concomitant loss of *Rac1* and *Rac2* impaired the development of a myeloproliferative disease and increased survival of mice transplanted with BCR-ABL1-expressing cells [57].

Constitutive phosphorylation of STAT1 and STAT5 has been reported in several *BCR-ABL1* positive cell lines [58] and primary CML cells [59], and seems to be independent of JAK. STAT5 can be directly activated by BCR-ABL1 [60] or indirectly through GRB2/RAS/RAC or HCK [56] to then up-regulate target genes, such as *CCND1* (leading to cell cycle progression) and the anti-apoptotic *BCL2L1*/*BCL-XL* [61, 62]. Although one study found that BCR-ABL1 induced a CML like disease in Stat5a/b$^{-/-}$ mice [63], another reported that complete deletion of Stat5a/b locus turned mice resistant to BCR-ABL1 transformation [64]. In addition, knock-down of STAT5 in primary CML cells blocks Ph + colony formation [62], and cells expressing a mutant BCR-ABL1 unable to activate STAT5 or wild type BCR-ABL1 with a dominant negative STAT5 are more apoptotic than wild type cells [65]. Altogether, these results support a role for STAT5 in BCR-ABL1 transformation.

BCR-ABL1 forms complexes with PI3K, CBL and the adapters CRK and CRKL [66], in which PI3K, and the downstream AKT1 and mTOR, are constitutively activated [67]. In addition, activation of RAS and the adapter GAB2 by GRB2 cause constitutive activation of PI3K [62]. PI3K exerts its oncogenic effects mainly by activation of mTOR, which forms the mTORC1 and mTORC2 complexes that play important roles in the proliferation and survival of BCR-ABL1-positive cells [49, 68]. PI3K activity is required for BCR-ABL1-mediated leukaemogenesis, since its inhibition impairs BCR-ABL1 transformation of HSCs [49, 67]. PI3K also hyperphosphorylates the transcription factor (TF) IRF8/ICSBP, preventing its DNA binding and reverting its transcriptional repression of the antiapoptotic *BCL2* gene [69].

AKT1 itself is an oncogene, and is essential for the resistance to apoptosis of BCR-ABL1-positive cells. It phosphorylates BAD, which promotes its sequestration by 14-3-3, and blocks its binding to BCL2 family members, consequently inhibiting apoptosis [70]. AKT1 also blocks apoptosis through phosphorylation of caspases [67], and downregulation of antiapoptotic BCL2L11/BIM [49].

Activation of MYC by BCR-ABL1 is dependent on the SH2 domain [71]. In addition, RAS/MAPK and PI3K/AKT1 pathways contribute to inducing *MYC* transcription or promoting MYC stability [67, 72]. Depending on the cellular context, MYC may transduce proliferative or apoptotic signals [67]; however, considering BCR-ABL1-mediated antiapoptotic mechanisms, the apoptotic arm of MYC is most likely inhibited in CML. Proliferation, on the other hand, may be induced by MYC's activation of cyclin and CDK transcription, repression of CIP/KIP family cyclin/CDK inhibitors' expression, and indirect induction of mTORC1 transcription [67].

7.3.3.2 Progression to Blast Crisis

CML progression is characterized by the occurrence of non-random chromosomal abnormalities. The most frequent are trisomy 8 (33 %), an additional Ph (30 %), isochromosome 17 (20 %), trisomy 19 (12 %), loss of the Y chromosome (8 % of males), trisomy 21 (7 %) and monosomy 7 (5 %) [73]. Although these changes are used as markers of disease progression, they may not necessarily be causal agents of transformation. Two important mechanisms and phenotypes related to the emergence of BC are addressed below.

Block in Differentiation

With progression of CML, the leukaemic clone undergoes differentiation arrest, resulting in a major increase of immature blasts at the expense of the terminally differentiated leucocytes. This differentiation arrest implies pathological interference with differentiation programmes involving the targeted activation/inactivation of tissue-specific genes by TF [74].

Abnormal CTNNB1/β-catenin signalling leads granulocyte-macrophage progenitors to acquire the stem cell-like capacity of unrestricted self-renewal [75]. In addition, interaction between CTNNB1 and BCR-ABL1 increases β-catenin transcriptional activity influencing leukaemic stem cell (LSC) lineage commitment as early as in CP, and loss of CTNNB1impairs the self-renewal of CML stem-cells [76, 77].

Another mechanism of differentiation arrest is the down-modulation of the TF CEBPA by BCR-ABL1, in BC but not in CP, through regulation of pre- and post-transcriptional mechanisms [78, 79]. CEBPA activates transcription of the *CSFR3*/*GCSFR* and *ID1* genes in myeloid cells, and its ectopic expression restores differentiation in BCR-ABL1-transformed cell lines or BC CML primary cells [80–83].

Additional causes of the block in differentiation in BC CML include mutations, translocations or deletions in genes that regulate differentiation and self-renewal of haematopoietic stem and progenitor cells, such as *GATA2* [84, 85], *RUNX1* [86–88], *ASXL1* [79, 87, 89], *IKZF1* [87, 90, 91] and *PAX5* [90, 92].

Genomic Instability

BCR-ABL1-transformed cell lines and CD34+ primary CML cells produce 2–6 times more ROS than the normal controls [93]. ROS can damage the DNA generating oxidized bases and double strand brakes (DSB). Accordingly, CD34+ CML cells accumulate three to eight times more oxidized bases and DSBs than normal cells [93]. At the same time, they display defective mismatch repair; stimulate DSBs repair but with low fidelity, through homologous recombination repair (HRR), non-homologous end-joining (NHEJ), and single strand annealing (SSA) repair mechanisms; and induce mutagenic nucleotide excision repair (NER), all of which

exacerbate genomic instability and contribute to disease progression (Fig. 7.5). The mechanisms of altered DNA repair in CML are addressed below.

ATR is a DNA damage 'sensor' that controls cell cycle check points. BCR-ABL1 was reported to translocate to the nucleus, following exposure to genotoxic agents, where it bound and inhibited ATR and CHEK1, allowing inappropriate DNA replication [95, 96]. In a contradictory study, however, ATR signalling was stimulated in BCR-ABL1-positive cells in response to genotoxic agents [97]. This result was further corroborated by recent findings that BCR-ABL1 inhibition reduces CHEK1 activation and cell cycle arrest in G2/M phase, and induces apoptosis in cells exposed to genotoxic agents [98]. Therefore, ATR signalling might contribute to chemotherapeutics resistance in CML.

The tumour suppressor BRCA1 is another 'sensor' that detects DNA damage and mediates cell cycle check points and HRR [99]. BRCA1 is virtually undetectable in CML cells and *BCR-ABL1*-transformed cell lines [100] and this absence contributes to the genomic instability observed in BCR-ABL1 cells [101]. To overcome BRCA1 deficiency, HRR occurs through the alternative RAD52-RAD51 pathway [102, 103].

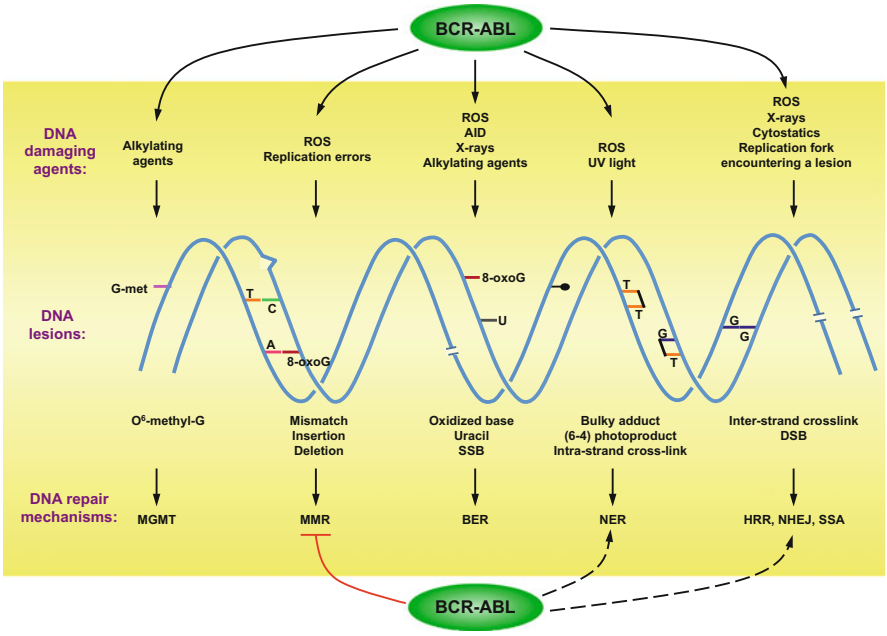

Fig. 7.5 BCR-ABL1 enhances DNA damage and deregulates DNA repair, the two main components of genomic instability. BCR-ABL1 positive cells accumulate more DNA lesions induced by endogenous and exogenous DNA genotoxic agents and, in parallel, activates cellular pathways which favour unfaithful DNA repair mechanisms. The overarching consequence of the two processes is the generation of improperly repaired DNA molecules containing point-mutations, insertions or deletions in genes which, once inappropriately expressed and/or activated, lead to the transformation into blast crisis (Figure modified from [94])

Both HRR and NHEJ promote less faithful ROS-induced DSB repair in *BCR-ABL1-transformed* cells [104]. Downregulation of PRKDC/DNA-PKcs, LIG4/DNA ligase IV and DCLRE1C/Artemis, and upregulation of LIG3/DNA ligase IIIα, WRN nuclease and RBBP8/CtIP in BCR-ABL1-positive cells may be responsible for the alternative error-prone NHEJ pathway observed in CML [105–108]. HRR, in turn, is abnormally stimulated to the detriment of its fidelity in CML due to BCR-ABL1-mediated overexpression and activation of RAD51, which promotes erroneous HRR when overstimulated [109–111]. Incorrect DNA repair can be prevented by mismatch repair, but BCR-ABL1 inhibits this process by abrogating heterodimerization of the mismatch repair proteins MLH1 and PMS2 [112]. SSA is a rare and unfaithful mechanism of DSB repair and BCR-ABL1 stimulates SSA activity in a dose-dependent manner and through up-regulation of *RBBP8*/*CtIP* [108, 113].

NER activity status in CML is controversial. In initial reports, BCR-ABL1 was found to interfere with NER proteins reducing NER activity [114, 115]. It was later suggested that $P210^{BCR-ABL}$ induced NER in myeloid but repressed it in lymphoid cell lines [116]. However, more recent findings reported no difference in NER activity between lymphoid and myeloid CML cell lines, and a BCR-ABL1 kinase-dependent increase in NER activity in CML cell lines [117].

Expression of BCR-ABL1 is also associated with upregulation of DNA polymerase β [118, 119], an enzyme involved in HRR, NER and base excision repair (BER) [120–122]. Due to its low-fidelity DNA repair, it might be expected that DNA polymerase β overexpression contributes to CML genomic instability. Accumulation of point mutations in CML might also result from BCR-ABL1 inhibition of UNG, the most active glycosylase during BER, in both CML primary and *BCR-ABL1*-transformed cells [123].

7.4 Targeted Therapy

The knowledge on BCR-ABL1 structure and function that accumulated over the past 30 years set up the scene for the design of 'molecularly targeted' therapy for CML. Since the tyrosine kinase activity of BCR-ABL1 is essential for disease development, it was the most attractive target for designer therapy, although not the only one approached [124–132]. Undoubtedly, the advent of TKIs, which block or prevent BCR-ABL1 oncogenic signalling, has been so far the most exciting and successful therapeutic advance in CML.

7.4.1 First Generation TKI: Imatinib

Imatinib mesilate (IM) is a small chemical compound which competes with ATP for binding to its pocket in the BCR-ABL1 kinase domain (KD), thus blocking the BCR-ABL1 oncogenic signal [45]. IM inhibits the kinase activity of all ABL1- and

ARG-containing proteins, the PDGFR family and the KIT receptor [133–135]. Such inhibition results in transcriptional modulation of various genes involved in the control of cell cycle, cell adhesion and cytoskeletal organization, leading the Ph + cell to an apoptotic death [44]. In addition, IM inhibits growth of CML primary cells and cell lines *in vitro* and *in vivo* [7, 8, 136].

In a phase I trial, IM showed little toxicity but proved to be highly effective [137]. The 8-year follow-up of the phase III IRIS trial reported an overall free survival rate (excluding discontinuation of therapy) of 85 % for CP CML patients under IM as first-line therapy, with 86 % of major molecular responses (MMR) [138]. In contrast, most of the responses of patients in BC are short-lived with very low (12–17 %) cytogenetic responses and median survival of 6.5–10 months [20].

7.4.1.1 Resistance to IM

While the efficacy of IM is unquestioned, resistance to TKIs became a pressing challenge in CML treatment. The persistence of minimal residual disease and, more worryingly, the development of refractoriness to single drug therapy, have dampened the initial enthusiasm. At the 8-year follow-up on the IRIS study, only 55 % of patients remained on IM therapy, and in 16 % of those who discontinued this was due to unsatisfactory therapeutic outcome [138]. Other studies have reported even higher resistance rates, varying from 12 to 50 % [19].

The definition of resistance can be based on its time of onset as primary resistance, i.e., failure to achieve a significant cytogenetic response, and secondary or acquired resistance, i.e., progressive reappearance of the leukaemic clone after an initial response to the drug. In addition, resistance can also be classified as BCR-ABL1-dependent and -independent. The first group encompasses the emergence of leukaemic clones with mutations in the BCR-ABL1 KD [139], overexpression of the BCR-ABL1 protein [140, 141] and amplification of the BCR-ABL1 oncogene [142, 143]. The mechanisms of BCR-ABL1-independent resistance include mostly defects in drug transport in and out of the leukaemic cells, and activation of oncogenic pathways downstream of BCR-ABL1 [144].

The most common mechanism for acquired IM resistance is through the development of point mutations in the ABL1 KD of BCR-ABL1 [144]. These mutations are not induced by the drug but, rather, confer resistance to rare populations of progenitors which are selected due to their capacity to survive and expand in the presence of the drug.

Mutations can be broadly categorized into four groups: (i) those which directly impair IM binding; (ii) those within the ATP binding site; (iii) those within the activation loop; and (iv) those within the catalytic domain (Fig. 7.6).

The substitution of isoleucine for threonine at position 315 of ABL1, or T315I, reduces the affinity for the drug by preventing the formation of a hydrogen bond between T315 and the secondary amino group of IM, and by sterically preventing the binding of IM [143]. Another amino acid that makes contact with IM is phenylalanine 317, and its mutation to leucine (F317L) also leads to resistance.

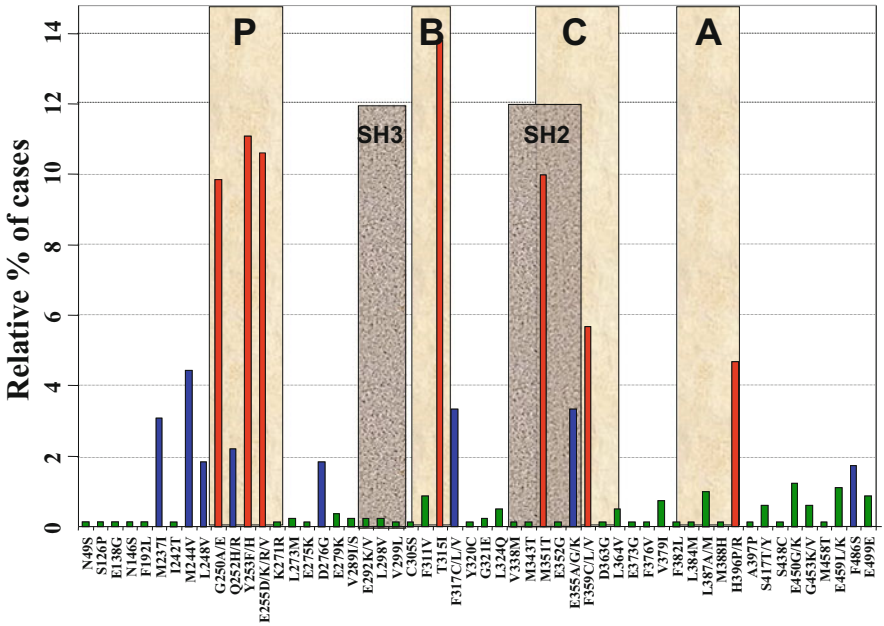

Fig. 7.6 Incidence of reported mutations within the kinase domain by percentage of total. The seven most frequent mutations are depicted in *red* and the following eight in *blue*; mutations shown in *green* have been reported in less than 2 % of clinical resistance cases. Specific regions of the kinase domain are indicated as P-loop or ATP binding site (*P*), imatinib binding site (*B*), catalytic domain (*C*) and activation loop (*A*). Also shown as SH2 and SH3 are the contact regions with SH2 and SH3 domain-containing proteins (Data based on [145, 146])

The ATP-binding loop (or P-loop) domain spans amino acids 248–256 [147]. Mutations in this domain are the most common and modify the flexibility of the P-loop destabilizing the conformation required for IM binding [148]. The most frequent of such mutations are substitutions at G250, Q252, Y253 and E255. An additional feature of clinical relevance is that IM-treated patients who harbour P-loop mutations have a worse prognosis than those with non-P-loop mutations [149–152].

The activation loop of the ABL1 kinase begins at amino acid 381 and can adopt a closed (inactive) or an open (active) conformation. IM forces ABL1 into the inactive conformation and is incapable of binding to the active one [153]. Mutations in the activation loop may disturb the energetic balance required to stabilize the closed conformation of the loop and, thus, favour the open conformation resulting in IM resistance [148].

Finally, some substitutions cluster in the catalytic domain (amino acids 350–363), a region that has a close topologic relation to the base of the activation loop. Therefore, mutations in this region can also influence IM binding [148].

The degree of IM resistance varies between mutations and is predicted to affect prognosis and response to treatment. Thus far, more than 100 different point mutations leading to a substitution of approximately 50 amino acids in the ABL1 KD have been identified in patients resistant to IM and this number is likely to increase with more sensitive methods of detection [154].

7.4.2 Second and Third Generation TKIs

7.4.2.1 Dasatinib

Dasatinib is a dual SRC/ABL1 kinase inhibitor that also binds to the ATP-binding site, but extends in the opposite direction from IM. It binds the inactive and active conformation of the ABL1 KD, has a greater affinity to this domain, and is more potent than IM [155]. In clinical trials, dasatinib showed significantly higher MMR and overall survival rates than IM for CP CML patients [156, 157]. CML patients in advanced phase also showed improved complete cytogenetic response (CCyR) rates under dasatinib; however, those are still low, at 32 % [158].

Dasatinib requires fewer contact points with ABL1 residues; therefore, it is active against several IM-associated mutations. The T315I and F317L mutations, however, lead to the least favourable responses [159–162]. Due to a direct interaction between F317 and dasatinib, several amino acid substitutions in this position result in dasatinib-resistant mutants, such as F317L, F317V, F317I, and F317S [150, 163]. In a phase III study of dasatinib in CP CML patients, development of mutations T315I, F317L, V299L, and, rarely, E255K correlated with loss of response [19].

7.4.2.2 Nilotinib

Nilotinib was designed as a chemical modification of IM and is 10–50 times more potent [164]. It also inhibits the activity of ARG, KIT, and PDGFRA and PDGFRB, but not SRC kinase. CP patients treated with nilotinib showed higher CCyR, MMR and overall survival rates, and lower transformation events than those under IM [19, 165, 166]. Moreover, in trials for patients with advanced CML, nilotinib treatment also resulted in higher CCyR rates than IM [19].

Similar to dasatinib, nilotinib inhibits the *in vitro* proliferation of most of the clinically relevant BCR-ABL1 mutants, except for the T315I [167–170]. Likewise, the degree of sensitivity/resistance to nilotinib also varies for individual mutants. Accordingly, the mutations T315I, E255K/V, F359C/V, and Y253H have shown association with lack of CCyR to nilotinib, followed by disease progression [170].

7.4.2.3 Bosutinib

Bosutinib is a potent second generation TKI that, like dasatinib, also has SRC inhibitory activity. In a phase III trial it showed higher MMR rates, and lower disease progression than IM [171]. Bosutinib also induces CCyR, albeit at a low rate (23 %), in patients resistant to IM or to either nilotinib or dasatinib [172]. At present, bosutinib is registered in many countries as a second- or third-line therapeutic agent.

7.4.2.4 Ponatinib

Ponatinib is a third generation TKI rationally designed to inhibit the T315I mutation, whilst still keeping activity against the unmutated and the majority of other BCR-ABL1 mutants. It also inhibits VEGFA, FGF, KIT and SRC kinases [173]. In a clinical trial of patients resistant or intolerant to nilotinib or dasatinib, or with the T315I mutation, ponatinib treatment caused CCyR and MMR in 46 % and 34 % of CP patients, respectively [174]. Moreover, 24 % of AP patients achieved CCyR and 16 % MMR, while only 18 % of BC patients experienced CCyR. Ponatinib's toxicity profile, however, can be a major drawback, since 5 % of patients suffered pancreatitis, and there was a significant association between ponatinib treatment and cardiovascular, cerebrovascular, and peripheral vascular events [174]. As a consequence, its indication is currently restricted to patients with a T315I mutation or for whom no other TKI is indicated.

7.4.2.5 Rebastinib

Rebastinib (or DCC-2036) is a switch pocket TKI rationally designed to induce an inactive conformation on BCR-ABL1. It retains full activity against the majority of BCR-ABL1 mutations, including T315I, but five P-loop mutants, G250E, Q252H, Y253H and E255K/V, in addition to F359I, have shown resistance to it [175, 176]. Preliminary results from a phase I trial (NCT00827138; www.clinicaltrials.gov) suggest it has anti-leukaemic activity in patients intolerant/refractory to other TKIs or positive for T315I [177], but a Phase II trial is not presently planned.

7.5 LSC as a Therapeutic Target in CML

Despite the success of TKI treatment, the persistence of minimal residual disease or the recurrence of disease upon cessation of therapy in most patients with undetectable BCR-ABL1, indicate that LSC persist even when response to treatment is optimal [178, 179].

Although primitive CML cells were shown to stop proliferating and enter a reversible cell cycle arrest upon IM treatment, they are resistant to TKI-induced

apoptosis both *in vitro* and *in vivo*, even when BCR-ABL1 signalling is effectively inhibited [180–188]. These results suggest that the LSCs are capable of surviving independently of BCR-ABL1.

It has been suggested that the LSC quiescent state was responsible for their resistance to IM. In fact, stimulating quiescent LSCs to enter the cell cycle with CSF3/G-CSF reduces the overall non-cycling cell population *in vitro* [189, 190]; however, in clinical practice, this does not impact on disease outcome [191]. Accordingly, even the cycling primitive CML cells resist apoptosis due to BCR-ABL1 inhibition [187]. Therefore, LSCs capacity to survive BCR-ABL1 inhibition may be mediated by their ability to escape apoptosis and/or to self-renew, or by interactions with the BM stroma [192]. Indeed, the resistance of primitive LSC is not confined to apoptosis induced by TKI but apparently extends to multiple pro-apoptotic agents, such as cytosine arabinoside and arsenic trioxide [183].

From the self-renewal aspect the Wnt/CTNNB1 and Hedgehog (Hh) pathways are altered in CML and are potential targets [193, 194]. For instance, knockout or pharmacological inhibition of either CTNNB1 or SMO in combination with TKI efficiently reduces LSC numbers *in vivo* and delays disease relapse [195, 196]. These data support the hypothesis that targeting self-renewal is effective to eradicate LSCs and is the basis of ongoing clinical trials with inhibitors of these pathways (NCT01606579, NCT01357655, NCT01218477, NCT01456676; www.clinicaltrials.gov).

It has been proposed that sequestration of LSCs in the BM niche induces the phenotype of environment-mediated drug resistance (EMDR) [197]. The mechanisms so far identified for EMDR include interaction of β1 integrins and CD44 with fibronectin on BM stromal cells, degradation of BCL2L11/BIM due to β1 integrin-mediated cell adhesion, activation of AKT1 through integrin-linked kinase, activation of JAK/STAT and HIF1A pathways, increase in STAT3 phosphorylation and subsequent expression of anti-apoptotic proteins, and interactions of CXCR4 in CML cells with extra cellular-matrix components and BM stromal cells [197, 198]. Special focus on CXCR4 as a possible drug target in CML has produced contradictory results with two studies showing that combination of CXCR4 antagonists with TKIs reduced leukaemia burden on CML mouse models [199, 200], while a third showed that combination of plerixafor with dasatinib had no advantage over dasatinib alone [201].

On a different approach, a farnesyltransferase inhibitor, BMS-214662, was found to selectively kill quiescent and dividing CML stem/progenitor cells *in vitro*, and its effect was enhanced when combined with either TKIs or a MEK inhibitor, PD184352, making it a promising agent for clinical development [202, 203].

Recent reports have also focused on manipulating the PP2A tumour suppressor activity to target LSCs. PP2A reactivation had been shown to effectively kill CML lines and primary cells from BC and both TKI-sensitive and -resistant patients [48, 204, 205]. Recently, the same group demonstrated that reactivating PP2A can erradicate quiescent LSCs, but not normal HSCs, through inhibition of the BCR-ABL1-JAK2-CTNNB1 signalling axis [206].

Other potential molecular targets in the CML LSC are listed on Table 7.1.

Table 7.1 Potential molecular targets on CML stem cells

Gene	Function	Effect on CML LSCs	Targeting agent[a]	Ref
ALOX5	Arachinonate 5-lipoxygenase	Activates CTNNB1, GATA1 and ZFPM1, therefore regulates LSC self-renewal and differentiation	5-LO inhibitor: Zileuton	[207]
BCL2 family	Anti-apoptotic	Promotes cell survival	Sabutoclax, ABT-737, omacetaxine	[208–210]
BCL6	Transcription factor (repressor)	Proto-oncogene, downstream target of FOXO3. Represses TP53 and CDKN2A/ARF and is essential for colony formation and leukaemia initiation	RI-BPI	[211]
BLK	SRC family tyrosine kinase	Tumour suppressor, induces CDKN1B/p27, inhibits LSC proliferation and delays CML development		[212]
CD27	TNF-receptor superfamily member	Promotes CML progression by inducing expression of Wnt target genes followed by proliferation and differentiation of LSCs	mAb against ligand CD70	[213]
CD44	Cell-surface glycoprotein, involved in adhesion and migration	Promotes homing and engraftment of LSCs	mAb	[214]
PTGS2/COX2	Cyclooxygenase-2, promotes prostaglandin biosysnthesis	Stabilizes CTNNB1 through prostaglanding E2 synthesis. Treatment of a CML mouse model with indomethacin reduced CTNNB1 expression and LSC numbers	Indomethacin	[196, 215]
FOXO3	Transcription factor	Key role in maintenance of LSCs by inhibiting apoptosis and promoting self-renewal		[216]
LGALS3/GAL3	Galectin family of carbohydrate binding proteins member	Activates AKT1 and MAPK, enhances clonogenic proliferation and homing of CML cells to the BM, and inhibits apoptosis		[217]
GSK3B	Serine-threonine kinase	Negative regulator of CTNNB1 and Hh pathway	SB216763 (inhibitor)	[218]
HIF1A	Transcription factor (activator), regulates hypoxia response	Essential for self-renewal of HSC and LSC; represses CDKN2A/INK4A (p16) and CDKN2A/ARF (p19)		[219, 220]
HSP90AA1/ HSP90	Molecular chaperone	Stabilizes BCR-ABL1	Retaspimycin (IPI-504)	[221]

(continued)

Table 7.1 (continued)

Gene	Function	Effect on CML LSCs	Targeting agent[a]	Ref
JAK2	Tyrosine kinase	Interacts with BCR-ABL1 through the adapter AHI1 in primitive CML progenitors	SAR302503, TG101209, ruxolitinib	[222]
MKNK1/MNK	Ser/Thr protein kinase family	Activates CTNNB1	CGP57380 (inhibitor)	[223]
MSR1	Macrophage scavenger receptor implicated in macrophage-associated processes	Suppresses LSC proliferation and CML development		[224]
PML	Transcription factor member of the tripartite motif (TRIM) family	Tumour suppressor, controls apoptosis, proliferation and senescence; represses mTOR. Essential for maintaining LSC quiescence	Arsenic trioxide	[225]
PP2A	Protein phosphatase 2A, Ser/Thr phosphatase	Inactivates JAK2 and CTNNB1, and thus hinders LSC survival and self-renewal	FTY720, OP449	[205, 206]
PTEN	Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase, dephosphorylates phosphoinositide substrates	Tumour suppressor in CML by reducing LSC number and function and consequently delaying CML development		[226]
RAD52	Recombinase, participates in HRR DSB repair	Promotes survival by repairing DSBs by an alternative HRR pathway in LSCs, which are BRCA-deficient	Peptide aptamer	[103]
SCD	Stearoyl-CoA desaturase	Involved in fatty acid biosynthesis. Tumour suppressor in LSCs with no effect in HSCs; regulates PTEN, TP53 and BCL2	Rosiglitazone (PPARγ agonist)	[227]
SELP (P-selectin)	Granule membrane protein, calcium-dependent receptor	Mediates cell-cell adhesion, is essential for haematopoiesis and its inhibition induces LSC self-renewal		[228–230]
SETBP1	SET nuclear oncogene protein binding	Cooperates with BCR-ABL1 conferring self-renewal capability to committed myeloid progenitors transforming them into LSCs in BC		[231]

SIRT1	NAD(+)-dependent histone/protein deacetylase	Promotes cell survival under different types of stress	Resveratrol, sirtinol, nicotinamide, tenovin-6	[232, 233]
SMO	Smoothened, frizzled class receptor, part of the hedgehog pathway	Represses NUMB, thus supports maintenance of CML LSC	SMO inhibitors: Cyclopamine, vismodegib, PF-04449913, BMS-833923, LDE-225, LEQ506, TAK-441	[193, 234–236]
TGFB1	Member of the TGFB family of cytokines	Suppresses AKT1 inhibition of FOXO3	LY364947 (inhibitor)	[216]

[a]Clinical drug or experimental compound/biological agent

7.6 Conclusion

The knowledge about the biology of CML increased exponentially since the Ph chromosome was first described. Even though this knowledge has led to the development of TKIs, which revolutionized CML treatment, there are still challenges to be overcome. Progression to BC, due to either primary failure to respond to a TKI or 'acquired' resistance, is still a major problem, since this aggressive disease stage is refractory to all types of available therapy. In addition, persistence of minimal residual disease in the majority of patients means they will have to continue under TKI therapy indefinitely. This raises two main problems, i.e., the risk that these patients develop resistance, which can then cause progression to BC, and the financial burden to families and/or Government's medical systems, which will have to provide lifelong expensive treatment for those patients. Therefore, there is still much to be investigated and learned about this apparently benign leukaemia before we can achieve the final goal of a cure for the great majority of patients.

References

1. Piller G (2001) Leukaemia – a brief historical review from ancient times to 1950. Br J Haematol 112:282–292
2. Nowell PC, Hungerford DA (1960) A minute chromosome in human chronic granulocytic leukemia. Science 132:1497
3. Rowley JD (1973) Letter: a new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. Nature 243:290–293
4. Shtivelman E, Lifshitz B, Gale RP, Canaani E (1985) Fused transcript of abl and bcr genes in chronic myelogenous leukaemia. Nature 315:550–554
5. Lugo TG, Pendergast AM, Muller AJ, Witte ON (1990) Tyrosine kinase activity and transformation potency of bcr-abl oncogene products. Science 247:1079–1082
6. Daley GQ, Van Etten RA, Baltimore D (1990) Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome. Science 247:824–830
7. Druker BJ, Tamura S, Buchdunger E, Ohno S, Segal GM et al (1996) Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. Nat Med 2:561–566
8. Deininger MWN, Goldman JM, Lydon NB, Melo JV (1997) The tyrosine kinase inhibitor CGP57148B selectively inhibits the growth of BCR-ABL positive cells. Blood 90:3691–3698
9. Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E et al (2001) Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N Engl J Med 344:1031–1037
10. Hoglund M, Sandin F, Hellstrom K, Bjoreman M, Bjorkholm M et al (2013) Tyrosine kinase inhibitor usage, treatment outcome, and prognostic scores in CML: report from the population-based Swedish CML registry. Blood 122:1284–1292
11. Mendizabal AM, Garcia-Gonzalez P, Levine PH (2013) Regional variations in age at diagnosis and overall survival among patients with chronic myeloid leukemia from low and middle income countries. Cancer Epidemiol 37:247–254

12. Bizzozero OJ Jr, Johnson KG, Ciocco A (1966) Radiation-related leukemia in Hiroshima and Nagasaki, 1946–1964. I. Distribution, incidence and appearance time. N Engl J Med 274:1095–1101
13. Landgren O, Goldin LR, Kristinsson SY, Helgadottir EA, Samuelsson J et al (2008) Increased risks of polycythemia vera, essential thrombocythemia, and myelofibrosis among 24,577 first-degree relatives of 11,039 patients with myeloproliferative neoplasms in Sweden. Blood 112:2199–2204
14. Hehlmann R, Heimpel H, Hasford J, Kolb HJ, Pralle H et al (1993) Randomized comparison of busulfan and hydroxyurea in chronic myelogenous leukemia: prolongation of survival by hydroxyurea. The German CML Study Group. Blood 82:398–407
15. Savage DG, Szydlo RM, Goldman JM (1997) Clinical features at diagnosis in 430 patients with chronic myeloid leukaemia seen at a referral centre over a 16-year period. Br J Haematol 96:111–116
16. Sawyers CL, Hochhaus A, Feldman E, Goldman JM, Miller CB et al (2002) Imatinib induces hematologic and cytogenetic responses in patients with chronic myelogenous leukemia in myeloid blast crisis: results of a phase II study. Blood 99:3530–3539
17. Vardiman JW, Melo JV, Baccarani M, Thiele J (2008) Chronic myelogenous leukaemia, BCR-ABL1 positive. In: Swerdlow SH, Campo E, Harris NL (eds) WHO classification of tumours of heaematopoietic and lymphoid tissues. International Agency for Research in Cancer (IARC), Lyon, pp 32–37
18. Wadhwa J, Szydlo RM, Apperley JF, Chase A, Bua M et al (2002) Factors affecting duration of survival after onset of blastic transformation of chronic myeloid leukemia. Blood 99:2304–2309
19. Radich JP (2012) Measuring response to BCR-ABL inhibitors in chronic myeloid leukemia. Cancer 118:300–311
20. Hehlmann R (2012) How I treat CML blast crisis. Blood 120:737–747
21. Melo JV, Gordon DE, Cross NC, Goldman JM (1993) The ABL-BCR fusion gene is expressed in chronic myeloid leukemia. Blood 81:158–165
22. Melo JV (1996) The diversity of BCR-ABL fusion proteins and their relationship to leukemia phenotype [editorial]. Blood 88:2375–2384
23. Melo JV, Myint H, Galton DA, Goldman JM (1994) P190BCR-ABL chronic myeloid leukaemia: the missing link with chronic myelomonocytic leukaemia? Leukemia 8:208–211
24. Ravandi F, Cortes J, AlBitar M, Arlinghaus R, Qiang GJ et al (1999) Chronic myelogenous leukaemia with p185(BCR/ABL) expression: characteristics and clinical significance. Br J Haematol 107:581–586
25. Pane F, Frigeri F, Sindona M, Luciano L, Ferrara F et al (1996) Neutrophilic-chronic myeloid leukemia (CML-N): a distinct disease with a specific molecular marker (BCR/ABL with C3/A2 junction). Blood 88:2410–2414
26. Gotlib J, Maxson JE, George TI, Tyner JW (2013) The new genetics of chronic neutrophilic leukemia and atypical CML: implications for diagnosis and treatment. Blood 122:1707–1711
27. Li S, Ilaria RL Jr, Million RP, Daley GQ, Van Etten RA (1999) The P190, P210, and P230 forms of the BCR/ABL oncogene induce a similar chronic myeloid leukemia-like syndrome in mice but have different lymphoid leukemogenic activity. J Exp Med 189:1399–1412
28. Deininger MW, Bose S, Gora TJ, Yan XH, Goldman JM et al (1998) Selective induction of leukemia-associated fusion genes by high-dose ionizing radiation. Cancer Res 58:421–425
29. Neves H, Ramos C, da Silva MG, Parreira A, Parreira L (1999) The nuclear topography of ABL, BCR, PML, and RARalpha genes: evidence for gene proximity in specific phases of the cell cycle and stages of hematopoietic differentiation [see comments]. Blood 93:1197–1207
30. Saglio G, Storlazzi CT, Giugliano E, Surace C, Anelli L et al (2002) A 76-kb duplicon maps close to the BCR gene on chromosome 22 and the ABL gene on chromosome 9: possible involvement in the genesis of the Philadelphia chromosome translocation. Proc Natl Acad Sci U S A 99:9882–9887

31. Sinclair PB, Nacheva EP, Leversha M, Telford N, Chang J et al (2000) Large deletions at the t(9;22) breakpoint are common and may identify a poor-prognosis subgroup of patients with chronic myeloid leukemia. Blood 95:738–743
32. Huntly BJ, Reid AG, Bench AJ, Campbell LJ, Telford N et al (2001) Deletions of the derivative chromosome 9 occur at the time of the Philadelphia translocation and provide a powerful and independent prognostic indicator in chronic myeloid leukemia. Blood 98:1732–1738
33. de la Fuente J, Merx K, Steer EJ, Muller M, Szydlo RM et al (2001) ABL-BCR expression does not correlate with deletions on the derivative chromosome 9 or survival in chronic myeloid leukemia. Blood 98:2879–2880
34. Quintas-Cardama A, Kantarjian H, Talpaz M, O'Brien S, Garcia-Manero G et al (2005) Imatinib mesylate therapy may overcome the poor prognostic significance of deletions of derivative chromosome 9 in patients with chronic myelogenous leukemia. Blood 105:2281–2286
35. Castagnetti F, Testoni N, Luatti S, Marzocchi G, Mancini M et al (2010) Deletions of the derivative chromosome 9 do not influence the response and the outcome of chronic myeloid leukemia in early chronic phase treated with imatinib mesylate: GIMEMA CML Working Party analysis. J Clin Oncol 28:2748–2754
36. Fialkow PJ, Martin PJ, Najfeld V, Penfold GK, Jacobson RJ et al (1981) Evidence for a multistep pathogenesis of chronic myelogenous leukemia. Blood 58:158–163
37. Biernaux C, Sels A, Huez G, Stryckmans P (1996) Very low level of major BCR-ABL expression in blood of some healthy individuals. Bone Marrow Transplant 17(Suppl 3):S45–S47
38. Bose S, Deininger M, Gora-Tybor J, Goldman JM, Melo JV (1998) The presence of typical and atypical BCR-ABL fusion genes in leukocytes of normal individuals: biologic significance and implications for the assessment of minimal residual disease. Blood 92:3362–3367
39. Pear WS, Miller JP, Xu L, Pui JC, Soffer B et al (1998) Efficient and rapid induction of a chronic myelogenous leukemia-like myeloproliferative disease in mice receiving P210 bcr/abl-transduced bone marrow. Blood 92:3780–3792
40. Zhang X, Ren R (1998) Bcr-Abl efficiently induces a myeloproliferative disease and production of excess interleukin-3 and granulocyte-macrophage colony-stimulating factor in mice: a novel model for chronic myelogenous leukemia. Blood 92:3829–3840
41. McWhirter JR, Galasso DL, Wang JY (1993) A coiled-coil oligomerization domain of Bcr is essential for the transforming function of Bcr-Abl oncoproteins. Mol Cell Biol 13:7587–7595
42. Pendergast AM, Quilliam LA, Cripe LD, Bassing CH, Dai Z et al (1993) BCR-ABL-induced oncogenesis is mediated by direct interaction with the SH2 domain of the GRB-2 adaptor protein. Cell 75:175–185
43. Pendergast AM, Muller AJ, Havlik MH, Maru Y, Witte ON (1991) BCR sequences essential for transformation by the BCR-ABL oncogene bind to the ABL SH2 regulatory domain in a non- phosphotyrosine-dependent manner. Cell 66:161–171
44. Deininger MW, Vieira S, Mendiola R, Schultheis B, Goldman JM et al (2000) BCR-ABL tyrosine kinase activity regulates the expression of multiple genes implicated in the pathogenesis of chronic myeloid leukemia. Cancer Res 60:2049–2055
45. Druker BJ (2008) Translation of the Philadelphia chromosome into therapy for CML. Blood 112:4808–4817
46. Tauchi T, Feng GS, Shen R, Song HY, Donner D et al (1994) SH2-containing phosphotyrosine phosphatase Syp is a target of p210bcr- abl tyrosine kinase. J Biol Chem 269:15381–15387
47. LaMontagne KR Jr, Flint AJ, Franza BR Jr, Pandergast AM, Tonks NK (1998) Protein tyrosine phosphatase 1B antagonizes signalling by oncoprotein tyrosine kinase p210 bcr-abl in vivo. Mol Cell Biol 18:2965–2975
48. Neviani P, Santhanam R, Trotta R, Notari M, Blaser BW et al (2005) The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR/ABL-regulated SET protein. Cancer Cell 8:355–368

49. Juan WC, Ong ST (2012) The role of protein phosphorylation in therapy resistance and disease progression in chronic myelogenous leukemia. Prog Mol Biol Transl Sci 106:107–142
50. Oda T, Heaney C, Hagopian JR, Okuda K, Griffin JD et al (1994) Crkl is the major tyrosine-phosphorylated protein in neutrophils from patients with chronic myelogenous leukemia. J Biol Chem 269:22925–22928
51. Pelicci G, Lanfrancone L, Salcini AE, Romano A, Mele S et al (1995) Constitutive phosphorylation of Shc proteins in human tumors. Oncogene 11:899–907
52. Cahill MA, Janknecht R, Nordheim A (1996) Signalling pathways: jack of all cascades. Curr Biol 6:16–19
53. Skorski T, Wlodarski P, Daheron L, Salomoni P, Nieborowska-Skorska M et al (1998) BCR/ABL-mediated leukemogenesis requires the activity of the small GTP- binding protein Rac. Proc Natl Acad Sci U S A 95:11858–11862
54. Raitano AB, Halpern JR, Hambuch TM, Sawyers CL (1995) The Bcr-Abl leukemia oncogene activates Jun kinase and requires Jun for transformation. Proc Natl Acad Sci U S A 92:11746–11750
55. Yang MY, Liu TC, Chang JG, Lin PM, Lin SF (2003) JunB gene expression is inactivated by methylation in chronic myeloid leukemia. Blood 101:3205–3211
56. Thomas EK, Cancelas JA, Zheng Y, Williams DA (2008) Rac GTPases as key regulators of p210-BCR-ABL-dependent leukemogenesis. Leukemia 22:898–904
57. Thomas EK, Cancelas JA, Chae HD, Cox AD, Keller PJ et al (2007) Rac guanosine triphosphatases represent integrating molecular therapeutic targets for BCR-ABL-induced myeloproliferative disease. Cancer Cell 12:467–478
58. Ilaria RL Jr, Van Etten RA (1996) P210 and P190(BCR/ABL) induce the tyrosine phosphorylation and DNA binding activity of multiple specific STAT family members. J Biol Chem 271:31704–31710
59. Chai SK, Nichols GL, Rothman P (1997) Constitutive activation of JAKs and STATs in BCR-Abl-expressing cell lines and peripheral blood cells derived from leukemic patients. J Immunol 159:4720–4728
60. Nieborowska-Skorska M, Wasik MA, Slupianek A, Salomoni P, Kitamura T et al (1999) Signal transducer and activator of transcription (STAT)5 activation by BCR/ABL is dependent on intact Src homology (SH)3 and SH2 domains of BCR/ABL and is required for leukemogenesis. J Exp Med 189:1229–1242
61. Horita M, Andreu EJ, Benito A, Arbona C, Sanz C et al (2000) Blockade of the bcr-Abl kinase activity induces apoptosis of chronic myelogenous leukemia cells by suppressing signal transducer and activator of transcription 5-dependent expression of bcl-x(L). J Exp Med 191:977–984
62. Quintas-Cardama A, Cortes J (2009) Molecular biology of bcr-abl1-positive chronic myeloid leukemia. Blood 113:1619–1630
63. Sexl V, Piekorz R, Moriggl R, Rohrer J, Brown MP et al (2000) Stat5a/b contribute to interleukin 7-induced B-cell precursor expansion, but abl- and bcr/abl-induced transformation are independent of stat5. Blood 96:2277–2283
64. Hoelbl A, Kovacic B, Kerenyi MA, Simma O, Warsch W et al (2006) Clarifying the role of Stat5 in lymphoid development and Abelson-induced transformation. Blood 107:4898–4906
65. Kantarjian HM, Giles F, Quintas-Cardama A, Cortes J (2007) Important therapeutic targets in chronic myelogenous leukemia. Clin Cancer Res 13:1089–1097
66. Sattler M, Salgia R, Okuda K, Uemura N, Durstin MA et al (1996) The proto-oncogene product p120CBL and the adaptor proteins CRKL and c-CRK link c-ABL, p190BCR/ABL and p210BCR/ABL to the phosphatidylinositol-3′ kinase pathway. Oncogene 12:839–846
67. Shortt J, Johnstone RW (2012) Oncogenes in cell survival and cell death. Cold Spring Harb Perspect Biol 4:a009829
68. Skorski T, Kanakaraj P, Nieborowska-Skorska M, Ratajczak MZ, Wen SC et al (1995) Phosphatidylinositol-3 kinase activity is regulated by BCR/ABL and is required for the growth of Philadelphia chromosome-positive cells. Blood 86:726–736

69. Middleton MK, Zukas AM, Rubinstein T, Jacob M, Zhu P et al (2006) Identification of 12/15-lipoxygenase as a suppressor of myeloproliferative disease. J Exp Med 203:2529–2540
70. del Peso L, Gonzalez-Garcia M, Page C, Herrera R, Nunez G (1997) Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science 278:687–689
71. Sawyers CL, Callahan W, Witte ON (1992) Dominant negative MYC blocks transformation by ABL oncogenes. Cell 70:901–910
72. Zou X, Rudchenko S, Wong K, Calame K (1997) Induction of c-myc transcription by the v-Abl tyrosine kinase requires Ras, Raf1, and cyclin-dependent kinases. Genes Dev 11:654–662
73. Johansson B, Fioretos T, Mitelman F (2002) Cytogenetic and molecular genetic evolution of chronic myeloid leukemia. Acta Haematol 107:76–94
74. Melo JV, Barnes DJ (2007) Chronic myeloid leukaemia as a model of disease evolution in human cancer. Nat Rev Cancer 7:441–453
75. Jamieson CH, Ailles LE, Dylla SJ, Muijtjens M, Jones C et al (2004) Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. N Engl J Med 351:657–667
76. Zhao C, Blum J, Chen A, Kwon HY, Jung SH et al (2007) Loss of beta-catenin impairs the renewal of normal and CML stem cells in vivo. Cancer Cell 12:528–541
77. Chen R, Hu T, Mahon GM, Tala I, Pannucci NL et al (2013) Ubiquitin-mediated interaction of p210 BCR/ABL with beta-catenin supports disease progression in a murine model for chronic myelogenous leukemia. Blood 122:2114–2124
78. Perrotti D, Cesi V, Trotta R, Guerzoni C, Santilli G et al (2002) BCR-ABL suppresses C/EBPalpha expression through inhibitory action of hnRNP E2. Nat Genet 30:48–58
79. Neviani P (2014) Genetic events other than BCR-ABL1. Curr Hematol Malig Rep 9:24–32
80. Smith LT, Hohaus S, Gonzalez DA, Dziennis SE, Tenen DG (1996) PU.1 (Spi-1) and C/EBP alpha regulate the granulocyte colony-stimulating factor receptor promoter in myeloid cells. Blood 88:1234–1247
81. Ferrari-Amorotti G, Keeshan K, Zattoni M, Guerzoni C, Iotti G et al (2006) Leukemogenesis induced by wild-type and STI571-resistant BCR/ABL is potently suppressed by C/EBPalpha. Blood 108:1353–1362
82. Guerzoni C, Bardini M, Mariani SA, Ferrari-Amorotti G, Neviani P et al (2006) Inducible activation of CEBPB, a gene negatively regulated by BCR/ABL, inhibits proliferation and promotes differentiation of BCR/ABL-expressing cells. Blood 107:4080–4089
83. Wagner K, Zhang P, Rosenbauer F, Drescher B, Kobayashi S et al (2006) Absence of the transcription factor CCAAT enhancer binding protein alpha results in loss of myeloid identity in bcr/abl-induced malignancy. Proc Natl Acad Sci U S A 103:6338–6343
84. Zhang SJ, Ma LY, Huang QH, Li G, Gu BW et al (2008) Gain-of-function mutation of GATA-2 in acute myeloid transformation of chronic myeloid leukemia. Proc Natl Acad Sci U S A 105:2076–2081
85. Zhang SJ, Shi JY, Li JY (2009) GATA-2 L359 V mutation is exclusively associated with CML progression but not other hematological malignancies and GATA-2 P250A is a novel single nucleotide polymorphism. Leuk Res 33:1141–1143
86. Mitani K, Ogawa S, Tanaka T, Miyoshi H, Kurokawa M et al (1994) Generation of the AML1-EVI-1 fusion gene in the t(3;21)(q26;q22) causes blastic crisis in chronic myelocytic leukemia. EMBO J 13:504–510
87. Grossmann V, Kohlmann A, Zenger M, Schindela S, Eder C et al (2011) A deep-sequencing study of chronic myeloid leukemia patients in blast crisis (BC-CML) detects mutations in 76.9 % of cases. Leukemia 25:557–560
88. Zhao LJ, Wang YY, Li G, Ma LY, Xiong SM et al (2012) Functional features of RUNX1 mutants in acute transformation of chronic myeloid leukemia and their contribution to inducing murine full-blown leukemia. Blood 119:2873–2882
89. Thorsteinsdottir U, Mamo A, Kroon E, Jerome L, Bijl J et al (2002) Overexpression of the myeloid leukemia-associated Hoxa9 gene in bone marrow cells induces stem cell expansion. Blood 99:121–129

90. Mullighan CG, Miller CB, Radtke I, Phillips LA, Dalton J et al (2008) BCR-ABL1 lymphoblastic leukaemia is characterized by the deletion of Ikaros. Nature 453:110–114
91. Suzuki K, Ono R, Ohishi K, Masuya M, Kataoka I et al (2012) IKAROS isoform 6 enhances BCR-ABL1-mediated proliferation of human CD34+ hematopoietic cells on stromal cells. Int J Oncol 40:53–62
92. Casolari DA, Makri M, Yoshida C, Muto A, Igarashi K et al (2013) Transcriptional suppression of BACH2 by the Bcr-Abl oncoprotein is mediated by PAX5. Leukemia 27:409–415
93. Skorski T (2012) Genetic mechanisms of chronic myeloid leukemia blastic transformation. Curr Hematol Malig Rep 7:87–93
94. Perrotti D, Jamieson C, Goldman J, Skorski T (2010) Chronic myeloid leukemia: mechanisms of blastic transformation. J Clin Invest 120:2254–2264
95. Costanzo V, Shechter D, Lupardus PJ, Cimprich KA, Gottesman M et al (2003) An ATR- and Cdc7-dependent DNA damage checkpoint that inhibits initiation of DNA replication. Mol Cell 11:203–213
96. Dierov J, Dierova R, Carroll M (2004) BCR/ABL translocates to the nucleus and disrupts an ATR-dependent intra-S phase checkpoint. Cancer Cell 5:275–285
97. Nieborowska-Skorska M, Stoklosa T, Datta M, Czechowska A, Rink L et al (2006) ATR-Chk1 axis protects BCR/ABL leukemia cells from the lethal effect of DNA double-strand breaks. Cell Cycle 5:994–1000
98. Kurosu T, Nagao T, Wu N, Oshikawa G, Miura O (2013) Inhibition of the PI3K/Akt/GSK3 pathway downstream of BCR/ABL, Jak2-V617F, or FLT3-ITD downregulates DNA damage-induced Chk1 activation as well as G2/M arrest and prominently enhances induction of apoptosis. PLoS One 8:e79478
99. Foulkes WD, Shuen AY (2013) In brief: BRCA1 and BRCA2. J Pathol 230:347–349
100. Deutsch E, Jarrousse S, Buet D, Dugray A, Bonnet ML et al (2003) Down-regulation of BRCA1 in BCR-ABL-expressing hematopoietic cells. Blood 101:4583–4588
101. Valeri A, Alonso-Ferrero ME, Rio P, Pujol MR, Casado JA et al (2010) Bcr/Abl interferes with the Fanconi anemia/BRCA pathway: implications in the chromosomal instability of chronic myeloid leukemia cells. PLoS ONE 5:e15525
102. Feng Z, Scott SP, Bussen W, Sharma GG, Guo G et al (2011) Rad52 inactivation is synthetically lethal with BRCA2 deficiency. Proc Natl Acad Sci U S A 108:686–691
103. Cramer-Morales K, Nieborowska-Skorska M, Scheibner K, Padget M, Irvine DA et al (2013) Personalized synthetic lethality induced by targeting RAD52 in leukemias identified by gene mutation and expression profile. Blood 122:1293–1304
104. Nowicki MO, Falinski R, Koptyra M, Slupianek A, Stoklosa T et al (2004) BCR/ABL oncogenic kinase promotes unfaithful repair of the reactive oxygen species-dependent DNA double-strand breaks. Blood 104:3746–3753
105. Deutsch E, Dugray A, AbdulKarim B, Marangoni E, Maggiorella L et al (2001) BCR-ABL down-regulates the DNA repair protein DNA-PKcs. Blood 97:2084–2090
106. Brady N, Gaymes TJ, Cheung M, Mufti GJ, Rassool FV (2003) Increased error-prone NHEJ activity in myeloid leukemias is associated with DNA damage at sites that recruit key nonhomologous end-joining proteins. Cancer Res 63:1798–1805
107. Sallmyr A, Tomkinson AE, Rassool FV (2008) Up-regulation of WRN and DNA ligase IIIalpha in chronic myeloid leukemia: consequences for the repair of DNA double-strand breaks. Blood 112:1413–1423
108. Salles D, Mencalha AL, Ireno IC, Wiesmuller L, Abdelhay E (2011) BCR-ABL stimulates mutagenic homologous DNA double-strand break repair via the DNA-end-processing factor CtIP. Carcinogenesis 32:27–34
109. Dubrez L, Eymin B, Sordet O, Droin N, Turhan AG et al (1998) BCR-ABL delays apoptosis upstream of procaspase-3 activation. Blood 91:2415–2422
110. Slupianek A, Schmutte C, Tombline G, Nieborowska-Skorska M, Hoser G et al (2001) BCR/ABL regulates mammalian RecA homologs, resulting in drug resistance. Mol Cell 8:795–806

111. Slupianek A, Dasgupta Y, Ren SY, Gurdek E, Donlin M et al (2011) Targeting RAD51 phosphotyrosine-315 to prevent unfaithful recombination repair in BCR-ABL1 leukemia. Blood 118:1062–1068
112. Stoklosa T, Poplawski T, Koptyra M, Nieborowska-Skorska M, Basak G et al (2008) BCR/ABL inhibits mismatch repair to protect from apoptosis and induce point mutations. Cancer Res 68:2576–2580
113. Cramer K, Nieborowska-Skorska M, Koptyra M, Slupianek A, Penserga ET et al (2008) BCR/ABL and other kinases from chronic myeloproliferative disorders stimulate single-strand annealing, an unfaithful DNA double-strand break repair. Cancer Res 68:6884–6888
114. Takeda N, Shibuya M, Maru Y (1999) The BCR-ABL oncoprotein potentially interacts with the xeroderma pigmentosum group B protein. Proc Natl Acad Sci U S A 96:203–207
115. Maru Y, Bergmann E, Coin F, Egly JM, Shibuya M (2001) TFIIH functions are altered by the P210BCR-ABL oncoprotein produced on the Philadelphia chromosome. Mutat Res 483:83–88
116. Canitrot Y, Falinski R, Louat T, Laurent G, Cazaux C et al (2003) p210 BCR/ABL kinase regulates nucleotide excision repair (NER) and resistance to UV radiation. Blood 102:2632–2637
117. Sliwinski T, Czechowska A, Szemraj J, Morawiec Z, Skorski T et al (2008) STI571 reduces NER activity in BCR/ABL-expressing cells. Mutat Res 654:162–167
118. Canitrot Y, Lautier D, Laurent G, Frechet M, Ahmed A et al (1999) Mutator phenotype of BCR–ABL transfected Ba/F3 cell lines and its association with enhanced expression of DNA polymerase beta. Oncogene 18:2676–2680
119. Canitrot Y, Laurent G, Astarie-Dequeker C, Bordier C, Cazaux C et al (2006) Enhanced expression and activity of DNA polymerase beta in chronic myelogenous leukemia. Anticancer Res 26:523–525
120. Canitrot Y, Hoffmann JS, Calsou P, Hayakawa H, Salles B et al (2000) Nucleotide excision repair DNA synthesis by excess DNA polymerase beta: a potential source of genetic instability in cancer cells. FASEB J 14:1765–1774
121. Hoeijmakers JH (2001) Genome maintenance mechanisms for preventing cancer. Nature 411:366–374
122. Canitrot Y, Capp JP, Puget N, Bieth A, Lopez B et al (2004) DNA polymerase beta overexpression stimulates the Rad51-dependent homologous recombination in mammalian cells. Nucleic Acids Res 32:5104–5112
123. Slupianek A, Falinski R, Znojek P, Stoklosa T, Flis S et al (2013) BCR-ABL1 kinase inhibits uracil DNA glycosylase UNG2 to enhance oxidative DNA damage and stimulate genomic instability. Leukemia 27:629–634
124. Quintas-Cardama A (2008) Experimental non-ATP-competitive therapies for chronic myelogenous leukemia. Leukemia 22:932–940
125. Rohon P (2012) Biological therapy and the immune system in patients with chronic myeloid leukemia. Int J Hematol 96:1–9
126. Kaymaz BT, Selvi N, Gokbulut AA, Aktan C, Gunduz C et al (2013) Suppression of STAT5A and STAT5B chronic myeloid leukemia cells via siRNA and antisense-oligonucleotide applications with the induction of apoptosis. Am J Blood Res 3:58–70
127. Kaymaz BT, Selvi N, Gunduz C, Aktan C, Dalmizrak A et al (2013) Repression of STAT3, STAT5A, and STAT5B expressions in chronic myelogenous leukemia cell line K-562 with unmodified or chemically modified siRNAs and induction of apoptosis. Ann Hematol 92:151–162
128. Koldehoff M, Zakrzewski JL, Beelen DW, Elmaagacli AH (2013) Additive antileukemia effects by GFI1B- and BCR-ABL-specific siRNA in advanced phase chronic myeloid leukemic cells. Cancer Gene Ther 20:421–427
129. Liu Y, Song Y, Ma W, Zheng W, Yin H (2013) Decreased microRNA-30a levels are associated with enhanced ABL1 and BCR-ABL1 expression in chronic myeloid leukemia. Leuk Res 37:349–356

130. Li Y, Wang H, Tao K, Xiao Q, Huang Z et al (2013) miR-29b suppresses CML cell proliferation and induces apoptosis via regulation of BCR/ABL1 protein. Exp Cell Res 319:1094–1101
131. Schurch CM, Riether C, Ochsenbein AF (2013) Dendritic cell-based immunotherapy for myeloid leukemias. Front Immunol 4:496
132. Valencia-Serna J, Gul-Uludag H, Mahdipoor P, Jiang X, Uludag H (2013) Investigating siRNA delivery to chronic myeloid leukemia K562 cells with lipophilic polymers for therapeutic BCR-ABL down-regulation. J Control Release 172:495–503
133. Buchdunger E, Zimmermann J, Mett H, Meyer T, Muller M et al (1996) Inhibition of the Abl protein-tyrosine kinase in vitro and in vivo by a 2-phenylaminopyrimidine derivative. Cancer Res 56:100–104
134. Okuda K, Weisberg E, Gilliland DG, Griffin JD (2001) ARG tyrosine kinase activity is inhibited by STI571. Blood 97:2440–2448
135. Savage DG, Antman KH (2002) Imatinib mesylate – a new oral targeted therapy. N Engl J Med 346:683–693
136. le Coutre P, Mologni L, Cleris L, Marchesi E, Buchdunger E et al (1999) In vivo eradication of human BCR/ABL positive leukemia cells with an ABL kinase inhibitor. J Natl Cancer Inst 91:163–168
137. Druker BJ, Talpaz M, Resta D, Peng B, Buchdunger E et al (1999) Clinical efficacy and safety of an Abl specific tyrosine kinase inhibitor as targeted therapy for chronic myelogenous leukemia [abstract]. Blood 94:368a
138. Deininger M, O'Brien SG, Guilhot F, Goldman JM, Hochhaus A et al (2009) International randomized study of interferon vs STI571 (IRIS) 8-year follow up: sustained survival and low risk for progression or events in patients with newly diagnosed chronic myeloid leukemia in chronic phase (CML-CP) treated with imatinib [abstract]. Blood (ASH Annual Meeting Abstracts) 114:1126
139. Gambacorti-Passerini CB, Gunby RH, Piazza R, Galietta A, Rostagno R et al (2003) Molecular mechanisms of resistance to imatinib in Philadelphia-chromosome-positive leukaemias. Lancet Oncol 4:75–85
140. Mahon FX, Deininger MW, Schultheis B, Chabrol J, Reiffers J et al (2000) Selection and characterization of BCR-ABL positive cell lines with differential sensitivity to the tyrosine kinase inhibitor STI571: diverse mechanisms of resistance. Blood 96:1070–1079
141. Weisberg E, Griffin JD (2000) Mechanism of resistance to the ABL tyrosine kinase inhibitor STI571 in BCR/ABL-transformed hematopoietic cell lines. Blood 95:3498–3505
142. le Coutre P, Tassi E, Varella-Garcia M, Barni R, Mologni L et al (2000) Induction of resistance to the Abelson inhibitor STI571 in human leukemic cells through gene amplification. Blood 95:1758–1766
143. Gorre ME, Mohammed M, Ellwood K, Hsu N, Paquette R et al (2001) Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. Science 293:876–880
144. Diamond JM, Melo JV (2011) Mechanisms of resistance to BCR-ABL kinase inhibitors. Leuk Lymphoma 52(Suppl 1):12–22
145. Apperley JF (2007) Part I: mechanisms of resistance to imatinib in chronic myeloid leukaemia. Lancet Oncol 8:1018–1029
146. Soverini S, Branford S, Nicolini FE, Talpaz M, Deininger MW et al (2014) Implications of BCR-ABL1 kinase domain-mediated resistance in chronic myeloid leukemia. Leuk Res 38:10–20
147. Hochhaus A, Kreil S, Corbin AS, La Rosee P, Muller MC et al (2002) Molecular and chromosomal mechanisms of resistance to imatinib (STI571) therapy. Leukemia 16:2190–2196
148. Shah NP, Nicoll JM, Nagar B, Gorre ME, Paquette RL et al (2002) Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia. Cancer Cell 2:117–125

149. Branford S, Rudzki Z, Walsh S, Parkinson I, Grigg A et al (2003) Detection of BCR-ABL mutations in patients with CML treated with imatinib is virtually always accompanied by clinical resistance, and mutations in the ATP phosphate-binding loop (P-loop) are associated with a poor prognosis. Blood 102:276–283
150. Soverini S, Colarossi S, Gnani A, Rosti G, Castagnetti F et al (2006) Contribution of ABL kinase domain mutations to imatinib resistance in different subsets of Philadelphia-positive patients: by the GIMEMA Working Party on Chronic Myeloid Leukemia. Clin Cancer Res 12:7374–7379
151. Nicolini FE, Corm S, Le QH, Sorel N, Hayette S et al (2006) Mutation status and clinical outcome of 89 imatinib mesylate-resistant chronic myelogenous leukemia patients: a retrospective analysis from the French intergroup of CML (Fi(phi)-LMC GROUP). Leukemia 20:1061–1066
152. Khorashad JS, Milojkovic D, Mehta P, Anand M, Ghorashian S et al (2008) In vivo kinetics of kinase domain mutations in CML patients treated with dasatinib after failing imatinib. Blood 111:2378–2381
153. Nagar B, Bornmann WG, Pellicena P, Schindler T, Veach DR et al (2002) Crystal structures of the kinase domain of c-Abl in complex with the small molecule inhibitors PD173955 and imatinib (STI-571). Cancer Res 62:4236–4243
154. Jabbour E, Soverini S (2009) Understanding the role of mutations in therapeutic decision making for chronic myeloid leukemia. Semin Hematol 46:S22–S26
155. Lombardo LJ, Lee FY, Chen P, Norris D, Barrish JC et al (2004) Discovery of N-(2-chloro-6-methyl- phenyl)-2-(6-(4-(2-hydroxyethyl)- piperazin-1-yl)-2-methylpyrimidin-4- ylamino) thiazole-5-carboxamide (BMS-354825), a dual Src/Abl kinase inhibitor with potent antitumor activity in preclinical assays. J Med Chem 47:6658–6661
156. Cortes JE, Jones D, O'Brien S, Jabbour E, Ravandi F et al (2010) Results of dasatinib therapy in patients with early chronic-phase chronic myeloid leukemia. J Clin Oncol 28:398–404
157. Kantarjian H, Shah NP, Hochhaus A, Cortes J, Shah S et al (2010) Dasatinib versus imatinib in newly diagnosed chronic-phase chronic myeloid leukemia. N Engl J Med 362:2260–2270
158. Kantarjian H, Cortes J, Kim DW, Dorlhiac-Llacer P, Pasquini R et al (2009) Phase 3 study of dasatinib 140 mg once daily versus 70 mg twice daily in patients with chronic myeloid leukemia in accelerated phase resistant or intolerant to imatinib: 15-month median follow-up. Blood 113:6322–6329
159. Cortes J, Jabbour E, Kantarjian H, Yin CC, Shan J et al (2007) Dynamics of BCR-ABL kinase domain mutations in chronic myeloid leukemia after sequential treatment with multiple tyrosine kinase inhibitors. Blood 110:4005–4011
160. Jabbour E, Kantarjian HM, Jones D, Reddy N, O'Brien S et al (2008) Characteristics and outcome of chronic myeloid leukemia patients with F317L BCR-ABL kinase domain mutation after therapy with tyrosine kinase inhibitors. Blood 112:4839–4842
161. Jabbour E, Kantarjian H, Jones D, Breeden M, Garcia-Manero G et al (2008) Characteristics and outcomes of patients with chronic myeloid leukemia and T315I mutation following failure of imatinib mesylate therapy. Blood 112:53–55
162. Muller MC, Cortes JE, Kim DW, Druker BJ, Erben P et al (2009) Dasatinib treatment of chronic-phase chronic myeloid leukemia: analysis of responses according to preexisting BCR-ABL mutations. Blood 114:4944–4953
163. Tokarski JS, Newitt JA, Chang CY, Cheng JD, Wittekind M et al (2006) The structure of Dasatinib (BMS-354825) bound to activated ABL kinase domain elucidates its inhibitory activity against imatinib-resistant ABL mutants. Cancer Res 66:5790–5797
164. Weisberg E, Manley PW, Breitenstein W, Bruggen J, Cowan-Jacob SW et al (2005) Characterization of AMN107, a selective inhibitor of native and mutant Bcr-Abl. Cancer Cell 7:129–141
165. Cortes JE, Jones D, O'Brien S, Jabbour E, Konopleva M et al (2010) Nilotinib as front-line treatment for patients with chronic myeloid leukemia in early chronic phase. J Clin Oncol 28:392–397

166. Larson RA, Hochhaus A, Hughes TP, Clark RE, Etienne G et al (2012) Nilotinib vs imatinib in patients with newly diagnosed Philadelphia chromosome-positive chronic myeloid leukemia in chronic phase: ENESTnd 3-year follow-up. Leukemia 26:2197–2203
167. Kantarjian H, Giles F, Wunderle L, Bhalla K, O'Brien S et al (2006) Nilotinib in imatinib-resistant CML and Philadelphia chromosome-positive ALL. N Engl J Med 354:2542–2551
168. Kantarjian H, O'Brien S, Talpaz M, Borthakur G, Ravandi F et al (2007) Outcome of patients with Philadelphia chromosome-positive chronic myelogenous leukemia post-imatinib mesylate failure. Cancer 109:1556–1560
169. le Coutre P, Ottmann OG, Giles F, Kim DW, Cortes J et al (2008) Nilotinib (formerly AMN107), a highly selective BCR-ABL tyrosine kinase inhibitor, is active in patients with imatinib-resistant or -intolerant accelerated-phase chronic myelogenous leukemia. Blood 111:1834–1839
170. Hughes T, Saglio G, Branford S, Soverini S, Kim DW et al (2009) Impact of baseline BCR-ABL mutations on response to nilotinib in patients with chronic myeloid leukemia in chronic phase. J Clin Oncol 27:4204–4210
171. Cortes JE, Kim DW, Kantarjian HM, Brummendorf TH, Dyagil I et al (2012) Bosutinib versus imatinib in newly diagnosed chronic-phase chronic myeloid leukemia: results from the BELA trial. J Clin Oncol 30:3486–3492
172. Cortes JE, Kantarjian HM, Brummendorf TH, Kim DW, Turkina AG et al (2011) Safety and efficacy of bosutinib (SKI-606) in chronic phase Philadelphia chromosome-positive chronic myeloid leukemia patients with resistance or intolerance to imatinib. Blood 118:4567–4576
173. O'Hare T, Shakespeare WC, Zhu X, Eide CA, Rivera VM et al (2009) AP24534, a pan-BCR-ABL inhibitor for chronic myeloid leukemia, potently inhibits the T315I mutant and overcomes mutation-based resistance. Cancer Cell 16:401–412
174. Cortes JE, Kim DW, Pinilla-Ibarz J, Le Coutre PD, Paquette R et al (2013) A phase 2 trial of ponatinib in Philadelphia chromosome-positive leukemias. N Engl J Med 369:1783–1796
175. Chan WW, Wise SC, Kaufman MD, Ahn YM, Ensinger CL et al (2011) Conformational control inhibition of the BCR-ABL1 tyrosine kinase, including the gatekeeper T315I mutant, by the switch-control inhibitor DCC-2036. Cancer Cell 19:556–568
176. Eide CA, Adrian LT, Tyner JW, Mac PM, Anderson DJ et al (2011) The ABL switch control inhibitor DCC-2036 is active against the chronic myeloid leukemia mutant BCR-ABLT315I and exhibits a narrow resistance profile. Cancer Res 71:3189–3195
177. Cortes JE, Talpaz M, Kantarjian HM, Smith H, Bixby D et al (2011) A phase 1 study o DCC-2036, a novel oral inhibitor of BCR-ABL kinase, in patients with Philadelphia chromosome positive (Ph+) leukemias including patients with T315I mutation. [abstract]. Blood (ASH Annual Meeting Abstracts) 118:601
178. Melo JV, Ross DM (2011) Minimal residual disease and discontinuation of therapy in chronic myeloid leukemia: can we aim at a cure? Hematology. Am Soc Hematol Educ Program 2011:136–142
179. Kinstrie R, Copland M (2013) Targeting chronic myeloid leukemia stem cells. Curr Hematol Malig Rep 8:14–21
180. Holyoake T, Jiang X, Eaves C, Eaves A (1999) Isolation of a highly quiescent subpopulation of primitive leukemic cells in chronic myeloid leukemia. Blood 94:2056–2064
181. Graham SM, Jorgensen HG, Allan E, Pearson C, Alcorn MJ et al (2002) Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 in vitro. Blood 99:319–325
182. Holtz MS, Slovak ML, Zhang F, Sawyers CL, Forman SJ et al (2002) Imatinib mesylate (STI571) inhibits growth of primitive malignant progenitors in chronic myelogenous leukemia through reversal of abnormally increased proliferation. Blood 99:3792–3800
183. Holtz MS, Forman SJ, Nonproliferating BR, CML (2005) CD34+ progenitors are resistant to apoptosis induced by a wide range of proapoptotic stimuli. Leukemia 19:1034–1041
184. Copland M, Hamilton A, Elrick LJ, Baird JW, Allan EK et al (2006) Dasatinib (BMS-354825) targets an earlier progenitor population than imatinib in primary CML but does not eliminate the quiescent fraction. Blood 107:4532–4539

185. Jorgensen HG, Allan EK, Jordanides NE, Mountford JC, Holyoake TL (2007) Nilotinib exerts equipotent anti-proliferative effects to imatinib and does not induce apoptosis in CD34+ CML cells. Blood 109:4016–4019
186. Konig H, Holtz M, Modi H, Manley P, Holyoake TL et al (2008) Enhanced BCR-ABL kinase inhibition does not result in increased inhibition of downstream signaling pathways or increased growth suppression in CML progenitors. Leukemia 22:748–755
187. Corbin AS, Agarwal A, Loriaux M, Cortes J, Deininger MW et al (2011) Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity. J Clin Invest 121:396–409
188. Hamilton A, Helgason GV, Schemionek M, Zhang B, Myssina S et al (2012) Chronic myeloid leukemia stem cells are not dependent on Bcr-Abl kinase activity for their survival. Blood 119:1501–1510
189. Jorgensen HG, Copland M, Allan EK, Jiang X, Eaves A et al (2006) Intermittent exposure of primitive quiescent chronic myeloid leukemia cells to granulocyte-colony stimulating factor in vitro promotes their elimination by imatinib mesylate. Clin Cancer Res 12:626–633
190. Holtz M, Forman SJ, Bhatia R (2007) Growth factor stimulation reduces residual quiescent chronic myelogenous leukemia progenitors remaining after imatinib treatment. Cancer Res 67:1113–1120
191. Drummond MW, Heaney N, Kaeda J, Nicolini FE, Clark RE et al (2009) A pilot study of continuous imatinib vs pulsed imatinib with or without G-CSF in CML patients who have achieved a complete cytogenetic response. Leukemia 23:1199–1201
192. Barnes DJ, Melo JV (2006) Primitive, quiescent and difficult to kill: the role of non-proliferating stem cells in chronic myeloid leukemia. Cell Cycle 5:2862–2866
193. Zhao C, Chen A, Jamieson CH, Fereshteh M, Abrahamsson A et al (2009) Hedgehog signalling is essential for maintenance of cancer stem cells in myeloid leukaemia. Nature 458:776–779
194. Crews LA, Jamieson CH (2013) Selective elimination of leukemia stem cells: hitting a moving target. Cancer Lett 338:15–22
195. Dierks C, Beigi R, Guo GR, Zirlik K, Stegert MR et al (2008) Expansion of Bcr-Abl-positive leukemic stem cells is dependent on Hedgehog pathway activation. Cancer Cell 14:238–249
196. Heidel FH, Bullinger L, Feng Z, Wang Z, Neff TA et al (2012) Genetic and pharmacologic inhibition of beta-catenin targets imatinib-resistant leukemia stem cells in CML. Cell Stem Cell 10:412–424
197. Kuroda J, Shimura Y, Yamamoto-Sugitani M, Sasaki N, Taniwaki M (2013) Multifaceted mechanisms for cell survival and drug targeting in chronic myelogenous leukemia. Curr Cancer Drug Targets 13:69–79
198. Li X, Miao H, Zhang Y, Li W, Li Z et al (2015) Bone marrow microenvironment confers imatinib resistance to chronic myelogenous leukemia and oroxylin A reverses the resistance by suppressing Stat3 pathway. Arch Toxicol 89:121–136
199. Weisberg E, Azab AK, Manley PW, Kung AL, Christie AL et al (2012) Inhibition of CXCR4 in CML cells disrupts their interaction with the bone marrow microenvironment and sensitizes them to nilotinib. Leukemia 26:985–990
200. Beider K, rash-Yahana M, Blaier O, Koren-Michowitz M, Abraham M et al (2014) Combination of imatinib with CXCR4 antagonist BKT140 overcomes the protective effect of stroma and targets CML in vitro and in vivo. Mol Cancer Ther 13:1155–1169
201. Agarwal A, Fleischman AG, Petersen CL, MacKenzie R, Luty S et al (2012) Effects of plerixafor in combination with BCR-ABL kinase inhibition in a murine model of CML. Blood 120:2658–2668
202. Copland M, Pellicano F, Richmond L, Allan EK, Hamilton A et al (2008) BMS-214662 potently induces apoptosis of chronic myeloid leukemia stem and progenitor cells and synergizes with tyrosine kinase inhibitors. Blood 111:2843–2853
203. Pellicano F, Simara P, Sinclair A, Helgason GV, Copland M et al (2011) The MEK inhibitor PD184352 enhances BMS-214662-induced apoptosis in CD34+ CML stem/progenitor cells. Leukemia 25:1159–1167

204. Neviani P, Santhanam R, Oaks JJ, Eiring AM, Notari M et al (2007) FTY720, a new alternative for treating blast crisis chronic myelogenous leukemia and Philadelphia chromosome-positive acute lymphocytic leukemia. J Clin Invest 117:2408–2421
205. Agarwal A, Mackenzie RJ, Pippa R, Eide CA, Oddo J et al (2014) Antagonism of SET using OP449 enhances the efficacy of tyrosine kinase inhibitors and overcomes drug resistance in myeloid leukemia. Clin Cancer Res 20:2092–2103
206. Neviani P, Harb JG, Oaks JJ, Santhanam R, Walker CJ et al (2013) PP2A-activating drugs selectively eradicate TKI-resistant chronic myeloid leukemic stem cells. J Clin Invest 123:4144–4157
207. Chen Y, Hu Y, Zhang H, Peng C, Li S (2009) Loss of the Alox5 gene impairs leukemia stem cells and prevents chronic myeloid leukemia. Nat Genet 41:783–792
208. Chen Y, Hu Y, Michaels S, Segal D, Brown D et al (2009) Inhibitory effects of omacetaxine on leukemic stem cells and BCR-ABL-induced chronic myeloid leukemia and acute lymphoblastic leukemia in mice. Leukemia 23:1446–1454
209. Robert F, Carrier M, Rawe S, Chen S, Lowe S et al (2009) Altering chemosensitivity by modulating translation elongation. PLoS ONE 4:e5428
210. Goff DJ, Court R, Sadarangani A, Chun HJ, Barrett CL et al (2013) A Pan-BCL2 inhibitor renders bone-marrow-resident human leukemia stem cells sensitive to tyrosine kinase inhibition. Cell Stem Cell 12:316–328
211. Hurtz C, Hatzi K, Cerchietti L, Braig M, Park E et al (2011) BCL6-mediated repression of p53 is critical for leukemia stem cell survival in chronic myeloid leukemia. J Exp Med 208:2163–2174
212. Zhang H, Peng C, Hu Y, Li H, Sheng Z et al (2012) The Blk pathway functions as a tumor suppressor in chronic myeloid leukemia stem cells. Nat Genet 44:861–871
213. Schurch C, Riether C, Matter MS, Tzankov A, Ochsenbein AF (2012) CD27 signaling on chronic myelogenous leukemia stem cells activates Wnt target genes and promotes disease progression. J Clin Invest 122:624–638
214. Krause DS, Lazarides K, von Andrian UH, Van Etten RA (2006) Requirement for CD44 in homing and engraftment of BCR-ABL-expressing leukemic stem cells. Nat Med 12:1175–1180
215. Goessling W, North TE, Loewer S, Lord AM, Lee S et al (2009) Genetic interaction of PGE2 and Wnt signaling regulates developmental specification of stem cells and regeneration. Cell 136:1136–1147
216. Naka K, Hoshii T, Muraguchi T, Tadokoro Y, Ooshio T et al (2010) TGF-beta-FOXO signalling maintains leukaemia-initiating cells in chronic myeloid leukaemia. Nature 463:676–680
217. Yamamoto-Sugitani M, Kuroda J, Ashihara E, Nagoshi H, Kobayashi T et al (2011) Galectin-3 (Gal-3) induced by leukemia microenvironment promotes drug resistance and bone marrow lodgment in chronic myelogenous leukemia. Proc Natl Acad Sci U S A 108:17468–17473
218. Reddiconto G, Toto C, Palama I, De Leo S, de Luca E et al (2012) Targeting of GSK3beta promotes imatinib-mediated apoptosis in quiescent CD34+ chronic myeloid leukemia progenitors, preserving normal stem cells. Blood 119:2335–2345
219. Wang Y, Liu Y, Malek SN, Zheng P, Liu Y (2011) Targeting HIF1alpha eliminates cancer stem cells in hematological malignancies. Cell Stem Cell 8:399–411
220. Zhang H, Li H, Xi HS, Li S (2012) HIF1alpha is required for survival maintenance of chronic myeloid leukemia stem cells. Blood 119:2595–2607
221. Peng C, Brain J, Hu Y, Goodrich A, Kong L et al (2007) Inhibition of heat shock protein 90 prolongs survival of mice with BCR-ABL-T315I-induced leukemia and suppresses leukemic stem cells. Blood 110:678–685
222. Chen M, Gallipoli P, DeGeer D, Sloma I, Forrest DL et al (2013) Targeting primitive chronic myeloid leukemia cells by effective inhibition of a new AHI-1-BCR-ABL-JAK2 complex. J Natl Cancer Inst 105:405–423

223. Lim S, Saw TY, Zhang M, Janes MR, Nacro K et al (2013) Targeting of the MNK-eIF4E axis in blast crisis chronic myeloid leukemia inhibits leukemia stem cell function. Proc Natl Acad Sci U S A 110:E2298–E2307
224. Chen Y, Sullivan C, Peng C, Shan Y, Hu Y et al (2011) A tumor suppressor function of the Msr1 gene in leukemia stem cells of chronic myeloid leukemia. Blood 118:390–400
225. Ito K, Bernardi R, Morotti A, Matsuoka S, Saglio G et al (2008) PML targeting eradicates quiescent leukaemia-initiating cells. Nature 453:1072–1078
226. Peng C, Chen Y, Yang Z, Zhang H, Osterby L et al (2010) PTEN is a tumor suppressor in CML stem cells and BCR-ABL-induced leukemias in mice. Blood 115:626–635
227. Zhang H, Li H, Ho N, Li D, Li S (2012) Scd1 plays a tumor-suppressive role in survival of leukemia stem cells and the development of chronic myeloid leukemia. Mol Cell Biol 32:1776–1787
228. Pelletier SD, Hong DS, Hu Y, Liu Y, Li S (2004) Lack of the adhesion molecules P-selectin and intercellular adhesion molecule-1 accelerate the development of BCR/ABL-induced chronic myeloid leukemia-like myeloproliferative disease in mice. Blood 104:2163–2171
229. Rossi DJ, Bryder D, Zahn JM, Ahlenius H, Sonu R et al (2005) Cell intrinsic alterations underlie hematopoietic stem cell aging. Proc Natl Acad Sci U S A 102:9194–9199
230. Sullivan C, Chen Y, Shan Y, Hu Y, Peng C et al (2011) Functional ramifications for the loss of P-selectin expression on hematopoietic and leukemic stem cells. PLoS ONE 6:e26246
231. Oakley K, Han Y, Vishwakarma BA, Chu S, Bhatia R et al (2012) Setbp1 promotes the self-renewal of murine myeloid progenitors via activation of Hoxa9 and Hoxa10. Blood 119:6099–6108
232. Yuan H, Wang Z, Li L, Zhang H, Modi H et al (2012) Activation of stress response gene SIRT1 by BCR-ABL promotes leukemogenesis. Blood 119:1904–1914
233. Li L, Wang L, Li L, Wang Z, Ho Y et al (2012) Activation of p53 by SIRT1 inhibition enhances elimination of CML leukemia stem cells in combination with imatinib. Cancer Cell 21:266–281
234. Zhang B, Irvine D, Ho YW, Buonamici M, Manley P et al (2010) Inhibition of chronic myeloid leukemia stem cells by the combination of the Hedgehog pathway inhibitor LDE225 with nilotinib [abstract]. Blood (ASH Annual Meeting Abstracts) 116:514
235. Jamieson C, Cortes JE, Oehler V, Baccaranni M, Kantarjian HM et al (2011) Phase 1 dose-escalation study of PF-04449913, an oral hedgehog (Hh) inhibitor, in patients with select hematologic malignancies [abstract]. Blood (ASH Annual Meeting Abstracts) 118:424
236. Katagiri S, Tauchi T, Okabe S, Minami Y, Kimura S et al (2013) Combination of ponatinib with Hedgehog antagonist vismodegib for therapy-resistant BCR-ABL1-positive leukemia. Clin Cancer Res 19:1422–1432

Chapter 8
Immunoglobulin and *MYC* Rearrangements in Multiple Myeloma Pathogenesis

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Contents

8.1 Multiple Myeloma Is a Post-germinal Centre Plasma Cell Tumour 140
8.2 Three Specific DNA Modifications Mediate Translocations in B Cell Tumours 141
8.3 *IGH* Rearrangements Are Present in About 50 % of MM Tumours 142
8.4 Primary *IGH* Translocations: An Early Oncogenic Event in ~40 % of MM Tumours 143
8.5 Primary *IGH* Breakpoints Generated by All 3 B Cell Specific DNA Modifications in MM 144
8.6 Locations of Breakpoints on Primary *IGH* Translocation Partner Chromosomes 145
8.7 Two Major Pathways of MM Pathogenesis: Primary *IGH* Translocations and Hyperdiploidy 146
8.8 *MYC* Rearrangements: A Paradigm for Secondary Rearrangements in MM 147
8.9 Complex *MYC* Rearrangements Mostly Hijack Nonrandom Super Enhancers 149
8.10 Characteristics of Secondary Immunoglobulin Rearrangements in Multiple Myeloma 151
8.11 Concluding Thoughts and Unresolved Issues 152
References 153

Abstract Multiple myeloma is a post-germinal centre plasma cell tumour that is usually preceded by a pre-malignant condition, monoclonal gammopathy of undetermined significance. Both are characterized by hyperdiploidy and recurrent immunoglobulin gene translocations that all result in the direct or indirect dysregulation of the CCND/RB1 pathway. Analysis of the translocation breakpoints suggests that they most frequently occur as a result of an error during class switch recombination, but also VDJ recombination, and sometimes somatic hypermutation. A rearrangement of the *MYC* locus is identified in nearly one half of untreated patients with MM, most frequently (>60 %) those with hyperdiploidy, and less frequently (<25 %) those with t(11;14). The rearrangements juxtapose *MYC* to super-enhancers from elsewhere in the genome, resulting in dysregulated expression of *MYC*. One-third of

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the rearrangements involve an immunoglobulin gene enhancer (*IGH>IGL>>IGK*), and two-thirds one of a variety of non-immunoglobulin gene enhancers that are frequently associated with plasma cell gene expression (e.g., *PRDM1, IGJ, FAM46C, TXNDC5, FOXO3*). It is likely that early rearrangements of the *MYC* locus cause the progression of monoclonal gammopathy to multiple myeloma in many patients, and that late rearrangements of the *MYC* locus, frequently involving an immunoglobulin gene enhancer, contribute to further, often extramedullary, tumour growth.

Keywords Multiple myeloma • Chromosome translocation • Plasma cell neoplasm • Monoclonal gammopathy of undetermined significance • MYC oncogene

8.1 Multiple Myeloma Is a Post-germinal Centre Plasma Cell Tumour

Multiple myeloma (MM) is a malignant tumour of post-germinal centre B cells, with a phenotype that is similar to long-lived bone marrow plasma cells (BMPCs) [1, 2]. MM mostly – perhaps always – is preceded by a pre-malignant MGUS (monoclonal gammopathy of undetermined significance) tumour, which is present in 4 % of individuals over the age of 50 [3, 4]. Presently there are no molecular or phenotypic markers that unequivocally distinguish MGUS and MM tumour cells, but MGUS can sporadically progress to symptomatic MM expressing the same monoclonal immunoglobulin at an average rate of ~1 % per year. Asymptomatic/smouldering MM (SMM) has a larger tumour mass than MGUS, but sporadically progresses to symptomatic MM at an average rage of ~10 % per year for the first 5 years, 3 % for the next 5 years, and 1 % for the next 10 years [5]. Unlike MGUS, therefore, the rate of progression of SMM is not fixed, but decreases over time. This indicates that SMM likely represents a mix of patients with MGUS, and others with MM who have not yet developed end-organ damage. Extramedullary MM, often manifested as primary or secondary plasma cell leukaemia (PCL), is a more aggressive stage of disease [2, 6]. MM cell lines (MMCLs) usually are generated only from extramedullary MM tumours [7].

MGUS and MM tumour cells secrete monoclonal immunoglobulin (Ig) that has a high prevalence of somatic mutations in the variable regions of the heavy and light chains, consistent with repeated rounds of somatic hypermutation (SHM) and antigen selection in precursor germinal centre B cells [8, 9]. Similar to long-lived BMPCs, most MGUS and MM tumours have undergone productive *IGH* class switch recombination (CSR), resulting mostly in expression of IgG or IgA and, rarely, IgE or IgD. However, about 1 % of tumours express IgM. In addition, about 15 % of MGUS and newly diagnosed MM tumours express an Ig light chain but no Ig heavy chain, most likely a consequence of post-germinal centre inactivation of *IGH* expression by a variety of mechanisms, including *IGH* translocations [4, 10].

8.2 Three Specific DNA Modifications Mediate Translocations in B Cell Tumours

Many kinds of B cell tumours have chromosomal translocations that involve the *IGH* locus (14q32.3), or less often one of the light chain loci: kappa (*IGK*, 2p12) or lambda (*IGL*, 22q11.2) [11]. Most of these translocations appear to be a consequence of errors in three B cell-specific DNA modification processes: (1) V(D)J recombination (VDJR), which is recombination activation gene complex (RAG1/RAG2) dependent, mostly occurs at very early stages of B cell development but sometimes during receptor editing or receptor revision at later stages of B cell development; (2) somatic hypermutation (SHM), which is activation-induced cytidine deaminase (AID) dependent, occurs almost exclusively in germinal centre B cells; and (3) IgH class switch recombination (CSR) which is also AID dependent and occurs mainly but not exclusively in germinal centre B cells (Table 8.1).

CSR involves recombination between long repetitive switch regions (Sμ, Sγ, Sα, Sε) located upstream of all *IGH* constant regions except for *IGH* delta [12]. The μ>δ CSR, involves Sμ and/or 422 bp duplicated sequences (σμ and σδ) positioned, respectively, upstream of Sμ and δ sequences [13, 14].

Each of these three mechanisms, which appear to be operative only at specific stages of B cell development, can generate double-strand breaks at or near specific sites in an *IG* locus, but sometimes can cause mutations or double-strand breaks in non-*IG* loci [11, 12, 15, 16]. The consequence of IG translocations typically is dysregulation and increased expression of an oncogene that is positioned near one or more of the strong *IGH* enhancers (Eμ, Eα1, Eα2), the *IGK* enhancers (E.IK, E.3′K), or the *IGL* enhancer (E.3′L) (Fig. 8.1).

Translocations mediated by the first two mechanisms typically generate a translocation breakpoint upstream of all enhancers, so that all enhancers are relocated to one of the two derivative chromosomes, der(14) for *IGH*, der(non-2) for *IGK*, and der(non-22) for *IGL*. By contrast, the CSR mechanism generates a breakpoint within or near a switch region, so that Eα1 and/or Eα2 are relocated to der(14) whereas Eμ (and sometimes Eα1) are relocated to der(non-14). As a result an oncogene can be dysregulated by an *IGH* enhancer on both chromosomes, as first demonstrated for *FGFR3* on der(14) and *WHSC1/MMSET* on der(4) in MM [17].

Table 8.1 B-cell specific DNA modifications that contribute to chromosome translocations in multiple myeloma

DNA modification	Mechanism	Timing
VDJ recombination	RAG1/RAG2	Mostly early B-cell, but sometimes during receptor editing or receptor revision in mature B cells
Somatic hypermutation	AID	Germinal centre B cells
Class switch recombination	AID	Mainly, but not exclusively, germinal centre B cells

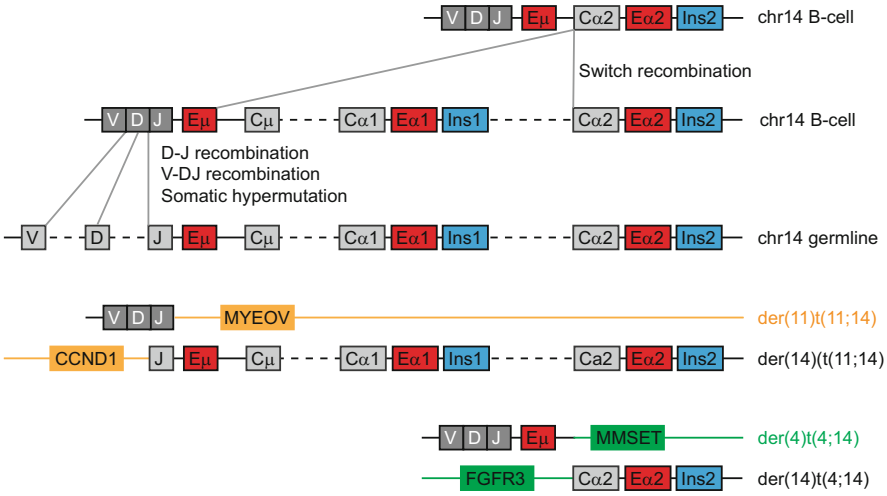

Fig. 8.1 Schema of *IGH* translocations mediated by B-cell specific DNA modifications in multiple myeloma. VDJ recombination, somatic hypermutation and class switch recombination remodel the germline *IGH* locus during B cell development (*top*). Errors in VDJ recombination and somatic hypermutation contribute to translocations that juxtapose all three *IGH* enhancers (*red*) to the translocated oncogene (e.g., CCND1) on der(14)t(11;14)(q13.3;q32.3) (*middle*). In contrast, errors in class switch recombination dissociate the *IGH* enhancers, so that the intronic enhancer (Eμ) dysregulates MMSET on der(4)t(4;14)(p16.3;q32.3), and the 3′ *IGH* enhancer (3′α2) dysregulates *FGFR3* on der(14)t(4;14)(p16.3;q32.3) (*bottom*). Insulators (*blue*) downstream of the 3′ enhancers prevent downstream gene dysregulation

8.3 *IGH* Rearrangements Are Present in About 50 % of MM Tumours

Conventional cytogenetics of MM greatly underestimated the prevalence of *IGH* translocations, and rarely identified the partner chromosomal loci, except for t(11;14). This was a consequence of: a low mitotic index, the telomeric location of the *IGH* locus together with telomeric or subtelomeric location of many partner loci, and the marked complexity of karyotypes [13, 18]. Interphase FISH assays largely solved the problem of detecting *IGH* translocations. Quantitative interphase FISH procedures are performed using a three colour cytoplasmic immunoglobulin method (cIg FISH) [19], or on purified MGUS/MM cells that are selected on anti-CD138 magnetic beads [20]. It is worth noting that the widely used Vysis *IGH* probes efficiently detect translocations but do not efficiently identify insertions of *IGH* sequences; fortunately other *IGH* probes that detect Ea1 and Ea2 sequences – including a Cytocel commercial probe – can efficiently detect both *IGH* translocations and *IGH* insertions [21]. Results from many studies indicate that the prevalence of *IGH* translocations is ~45 % for MGUS tumours, ~55 % for intramedullary MM tumours, ~80 % in primary PCL, and ~80 % in MMCL [6, 20, 22–27]. There is very

little information about *IGK* or *IGL* rearrangements. However, *IGL* rearrangements were identified in about 15–20 % of MMCL or advanced MM tumours, whereas IGK rearrangements are much less frequent, i.e., ~2 % of MMCL or advanced MM tumours [27].

8.4 Primary *IGH* Translocations: An Early Oncogenic Event in ~40 % of MM Tumours

Primary *IGH* translocations are defined as recurrent translocations that are present in both MGUS and MM tumours, although it is unknown if they represent the initial oncogenic event [28]. The seven recurrent *IGH* translocations comprise three translocation groups, with the chromosomal site, target oncogene(s), and approximate prevalence in MM as indicated [1, 20, 25, 29–31]:

Cyclin D group:	
11q13.3 (*CCND1*)	15 %
12p13.3 (*CCND2*)	~1 %
6p21.1 (*CCND3*)	2 %
MAF group:	
16q23 (*MAF*)	5 %
20q12 (*MAFB*)	2 %
8q24.3 (*MAFA*)	<1 %
WHSC1/MMSET and FGFR3 group:	
4p16.3 (*WHSC1/MMSET* and *FGFR3*)	14 %

Together the combined prevalence of primary *IGH* translocations is approximately 40 % in MM but somewhat lower in MGUS. Compared to MM, MGUS has a significant decrease in the prevalence of t(4;14) (~4 %) and possibly t(14;16), and an apparent increase in t(11;14). It is possible but unproven that the lower prevalence of t(4;14) and t(14;16) in MGUS results from a more rapid progression from MGUS to MM, and vice versa for the increased prevalence of t(11;14) in MGUS. Surprisingly, the prevalence of t(11;14) is markedly increased (~40 %) for MGUS tumours that are associated with primary amyloidosis, but presently there is not a good explanation for this observation [32, 33]. Most of the primary *IGH* translocations appear to be generated as simple reciprocal translocations, usually with both derivative chromosomes present at all disease stages. However, for the t(4;14), about 20 % of MM tumours and MMCL have lost the der(14) so that *FGFR3* no longer is dysregulated [34–36]. There are no examples of MM tumours or MMCL that have lost the der(4). This suggests that dysregulation of *MMSET* on the der(4) is essential both for early pathogenesis but also for maintenance of the tumour throughout the course of the disease. Some MM tumours and MMCLs with the t(11;14) do not have the der (11), and others have two copies of the der(14) and one copy of the der(11) [27, 37, 38]. There are very few examples of insertions that juxtapose *IGH* enhancer sequences

with one of the seven recurrent *IGH* partners [27]. Rare monoclonal tumours have two independent *IGH* translocations or insertions that involve two different *IGH* translocation groups, e.g., 4p16.3 and 11q13.3; 4p16.3 and 16q23; 6p21.1 and 16q23; and possibly 11q13.3 plus 20q12 [24, 27]. Curiously, *IGL* and IGK translocations rarely involve one of the seven recurrent *IGH* partners, but *IGL* translocations involving 6p21.1 [39] or 16q23 [40], and insertions involving 20q12 and *IGK* [41] or *IGL* [27] have been identified.

8.5 Primary *IGH* Breakpoints Generated by All 3 B Cell Specific DNA Modifications in MM

Metaphase and interphase FISH analyses, together with conventional cytogenetics and spectral karyotypic (SKY) analyses, can efficiently identify most IG rearrangements, including the apparent partner chromosomal loci. However, identification of the rearrangement breakpoints and target genes has been a more involved process. Initially this was done by a combination of Southern blotting to map breakpoints, plus conventional cloning or various PCR procedures to isolate more than 50 primary *IGH* breakpoints [reviewed in [28]]. Unfortunately, this was a somewhat biased approach, which focused mainly on *IGH* switch regions, and to a somewhat lesser extent on sequences between JH and Sμ. More recently, mate pair sequencing (PLB&WMK, unpublished), whole genome sequencing [42], and targeted sequencing of captured genomic regions containing *IG* sequences [43] have resulted in more than 60 additional primary *IGH* breakpoints that are less biased for specific regions within the *IGH* locus. A summary from the analysis of more than 100 primary *IGH* breakpoints includes the following [18, 28, 43]. First, the locations of breakpoints in the *IGH* locus suggest that all three B cell specific DNA modification processes (CSR>VDJR>SHM) can be implicated (Table 8.1). Second, the positioning of breakpoints near or within V, D, or J sequences suggests errors in D>J joining and less often V>DJ joining or V region replacement, all of which are mediated by the VDJR mechanism. Third, two t(14;16) breakpoints are located near one of two nearly identical non-adjacent sequences in the *IGH* locus but with no homology to the partner chromosome; the authors suggested a homologous recombination mechanism, but the molecular basis for this event is unclear [43]. Fourth, in all cases, the t(4;14) involves *IGH* switch regions. Fifth, about half of the t(11;14) breakpoints occur near or within switch regions, about one third at locations suggesting errors in VDJR, and most of the others at locations consistent with errors in SHM [18, 28, 43]. Sixth, there is less data for the t(6;14), t(14;16), and t(14;20) breakpoints, but it appears that about 50 % are located near or within switch regions.

The location of breakpoints within the *IGH* locus has been used to infer not only the occurrence of an error in a particular B cell specific DNA modification process but also the time of occurrence during B cell development. The latter makes two assumptions: (1) that we know at what stage(s) of normal B cell development a

particular modification occurs; and (2) that a particular modification occurs only during the stage(s) implicated for normal B cell development, but not at another stage in a pathological situation. For translocations involving switch regions there are several lines of evidence indicating that most – if not all – of these rearrangements occur during normal *IGH* switch recombination. First, cloning and sequencing of *IGH* switch breakpoints in 40 t(4;14) MM tumours or MMCLs show that the 5′ switch breakpoint on the der(4) always is μ-, μγ-, or μα- and the 3′ switch breakpoint on the der(14) involves -μ, -γ, or -α, and less often –μγ or -μα; one caveat is that t(4;14) rearrangements may be uniquely constrained to have breakpoints close to Eμ so that *MMSET* can be dysregulated on the der(4) [28]. Second, in the U266 MMCL, a productive μ>ε switch was associated with a second switch event that excised a portion of the intervening switch sequences (including Eα1), and inserted them near the *CCND1* gene at 11q13.3 [44]. Third, when 3′ switch breakpoints involve distal switch regions (-γ2, -γ4, -α2), Eα1 almost always is deleted as would be predicted if a normal switch event involved Sμ and a distal switch region [28].

The timing of translocations that appear to be mediated by errors in VDJR or SHM presents a more complex situation. It was suggested that rearrangements involving V segments associated with JH mutations could represent either receptor revision or SHM in germinal centre B cells, whereas the lack of JH mutations was more consistent with revision in B cells that have not yet undergone SHM [43]. Most interestingly, the authors described seven examples [six with the t(11;14)] of translocation breakpoints suggesting an apparent error in D>J joining. As a result they suggested that some translocations can occur in pro-B cells that are undergoing D>J rearrangements. This provocative result is consistent with the fact that Eμ. CCND1 transgenic mice show only minimal abnormalities of normal B cell development [45, 46]. However, an alternative explanation that D>J joining can occur in mature human B cells – perhaps only as a rare pathological event – cannot be excluded.

8.6 Locations of Breakpoints on Primary *IGH* Translocation Partner Chromosomes

The primary *IGH* translocation partner chromosome breakpoints have a number of interesting features [28, 43]:

1. The breakpoints can be located more than 1000 kb centromeric to the oncogene that is dysregulated on the der(14), e.g., 20q12 breakpoints are 300–1200 kb downstream of *MAFB* and 16q23 breakpoints are 480–1280 kb downstream of *MAF*.
2. The 16q23 breakpoints are located mostly within the ultimate intron of *WWOX*, which includes the FRA16D fragile site, and has been proposed to be a tumour suppressor gene in some tumours.

3. For two translocations the oncogene apparently can be dysregulated even when other genes are closer to the breakpoint. For t(4;14) tumours, *FGFR3* is dysregulated, whereas *LETM1*, which is located between *FGFR3* and the breakpoint, is not dysregulated. For t(11;14) tumours, the breakpoints are mostly scattered throughout the 400 kb region upstream of *CCND1* and downstream of *MYEOV*. However, breakpoints in five tumours were located in a 90 kb region upstream of *MYEOV*, and the breakpoint in one tumour was located in the *PPP6R3* gene, which is located centromeric to both *TPCN2* and *MYEOV* [43].
4. The mechanism(s) responsible for creating double-stranded breaks on the partner chromosomes are not well understood; However, the frequent involvement of AID was suggested based on the presence of CpG dinucleotide sequences or WGCW sequences, known AID recognition sites, near the breakpoint in up to 61 % of samples [43].
5. The breakpoints in t(4;14) tumours are invariably located upstream of *MMSET* or clustered in 5′ introns in *MMSET* [17, 35, 43]. Therefore *FGFR3* is positioned 30–80 kb telomeric to the 3′*IGH* enhancers on the der(14). Full length or variously truncated portions of *MMSET* are positioned near the Eμ enhancer on the der(4), with consequent expression of hybrid transcripts that contain JH or Iμ exons. Importantly, the hybrid transcripts, unique for this translocation, can be detected by a very specific and sensitive PCR assay. This invariant scenario is consistent with the need to simultaneously dysregulate both *FGFR3* and *MMSET* early in tumourigenesis, despite the fact that the der(14) is not present in ~20 % of MM tumours, presumably because subsequent oncogenic events (e.g. *RAS* gene mutations) obviate the need for dysregulation of *FGFR3*.

8.7 Two Major Pathways of MM Pathogenesis: Primary *IGH* Translocations and Hyperdiploidy

Approximately half of MM tumours are hyperdiploid (HRD), typically containing 48–60 chromosomes and, typically with a characteristic pattern of multiple trisomies involving eight chromosomes (3, 5, 7, 9, 11, 15, 19, 21) [47, 48]. Non-hyperdiploid (NHRD) MM tumours usually are hypodiploid, pseudodiploid, or subtetraploid, often with near diploid and subtetraploid cells in the same tumour. Interphase FISH studies indicate that HRD tumours constitute a somewhat lower fraction in MGUS compared to MM [20, 49]. Primary *IGH* translocations are present in ~70 % of NHRD MM tumours but only ~15 % of HRD MM tumours. Similar to MM, primary *IGH* translocations are found mostly in NHRD MGUS tumours [20]. Despite some overlap, it generally is thought that the two major pathways of MGUS/MM pathogenesis are associated with primary *IGH* translocations or hyperdiploidy, respectively, as early if not initiating events. The causes and molecular consequences of hyperdiploidy have not been elucidated at this time. However, it is notable that the fraction of MM tumours with hyperdiploidy increases with age and the fraction of tumours with *IGH* translocations decreases with age [50].

8.8 *MYC* Rearrangements: A Paradigm for Secondary Rearrangements in MM

Prior to the development of FISH and spectral karyotyping technology, conventional cytogenetic analysis rarely identified *MYC* rearrangements in MM tumours or MMCLs [18]. The finding that only one *MYC* allele was expressed in all nine informative MMCLs examined prompted metaphase FISH studies, which showed that 19 of 20 MMCLs and 7 of 14 advanced MM tumours had complex rearrangements of *MYC* or *MYCL*, many of which did not involve one of the three *IG* loci [40]. Collectively, comprehensive metaphase FISH studies, which utilized *IGH*, *IGL, IGK, MYC*, *MYCL*, *MYCN*, and whole chromosome painting probes, identified rearrangements of *MYC*, *MYCN*, or *MYCL* in 42/53 (79 %) MMCLs and 28/62 (45 %) advanced MM tumours [27, 40]. There was heterogeneity of *MYC* rearrangements in some MM tumours. Less comprehensive spectral karyotyping studies detected a somewhat lower prevalence of *MYC* rearrangements in advanced MM tumours [37, 38]. The *MYC* rearrangements were mostly unbalanced translocations or insertions, often with involvement of three chromosomes, and sometimes with associated amplification, duplication, deletion, or inversion (Fig. 8.2).

Interphase FISH studies using *IGH*, *IGL*, and *MYC* probes, together with a pair of probes flanking the *MYC* locus, were done on 22 MMCL, 529 newly diagnosed MM tumours, 53 relapsed MM tumours, and 63 MGUS tumours [51]. *MYC* locus rearrangements, which often were present in only a fraction of primary tumour cells, were detected in 55 % of MMCL and 16 % of MM tumours, but only 3 % of MGUS tumours. Another study reported a smouldering MM tumour with no *MYC* rearrangement that progressed to symptomatic MM with a *MYC* rearrangement [52]. Finally, in contrast to primary *IGH* translocations (above), the *IGH* breakpoints in *MYC*:*IGH* translocations are rarely within or near switch regions or VDJ sequences [28, 29]. Therefore, they are unlikely to be caused by errors in the B cell specific DNA modification mechanisms that are thought to be inactive in plasma cells and plasma cell tumours. Together, these results indicate that *MYC* rearrangements are a paradigm for secondary rearrangements that are uncommon in MGUS but have an increasing prevalence during progression of MM.

The combination of metaphase FISH (see above) and Agilent 244 K CGH arrays detected *MYCN* (one MMCL), *MYCL* (one MMCL) or *MYC* locus (chr8: 126000000–130000000[hg19]) rearrangements in 47/53 (89 %) MMCL [29]. Rearrangements in five MMCLs were detected only by FISH and in five other tumours only by CGH. For the 47 MMCL with *MYC* rearrangements, 28 (60 %) repositioned a *MYC* family member near one of the *IG* 3′ enhancer sequences that are included in the FISH probes (21 with *IGH*, 5 with *IGL*, and 2 with *IGK*).

A combination of 244 K CGH, interphase FISH to detect *MYC*:*IGH* and *MYC*:*IGL* fusions, and gene expression profiling identified *MYC* locus rearrangements or *MYCN* expression (two tumours) in 108/218 (49 %) MM tumours [29]. The prevalence of rearrangements was not significantly different for treated (52 %) vs newly diagnosed (46 %) vs smouldering (55 %) MM tumours. Of these, 33/218 (15 %)

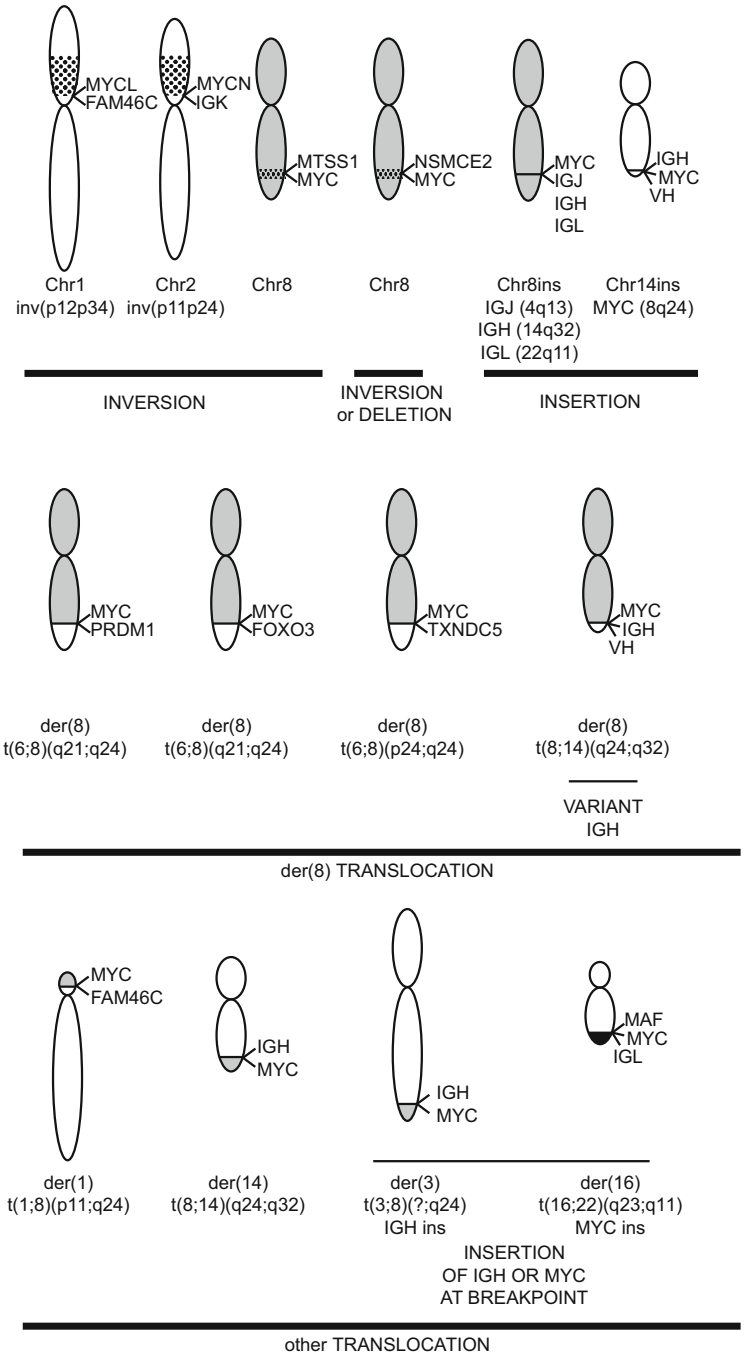

Fig. 8.2 Rearrangements of *MYC*, *MYCN* and *MYCL* in multiple myeloma. Ideograms of chromosomal rearrangements including inversions (*stippled*), deletions (*stippled*), insertions (*black line*), and translocations (chr8 is shaded in *grey*). A variant *IGH* translocation has a small inversion near the breakpoint so that the insulator is not positioned between *MYC* and the 3′ *IGH* enhancer

tumours had *MYC*:*IGH* (11 %) or *MYC*:*IGL* (4 %) fusions, with 22 of these tumours also having a rearrangement detected by CGH. There was significant heterogeneity of the *MYC*:*IG* fusion signal in 6 of the 33 tumours. Using *MYC* as a reference location, the *MYC* locus copy number abnormalities detected by CGH, which were similar for MMCL and MM tumours, comprised four groups: centromeric changes (mostly segmental loss), segmental *MYC* gain, telomeric segmental gain that was located mostly in a region 350–500 kb downstream of *MYC*, and other telomeric CNA. *MYC*:*IGH* or *MYC*:*IGL* fusions were present in only 4 % of the tumours with centromeric changes, but in about 30 % of the tumours in each of the other three groups.

MYC expression in MMCL and MM tumours was mostly monoallelic with rearrangements, but mostly biallelic without rearrangements. The mean level of *MYC* RNA expression was about threefold higher in tumours or MMCL with vs. those without *MYC* rearrangements. Surprisingly, for both MMCL and MM tumours, the mean level of *MYC* expression was similar for rearrangements that did or did not involve *IG* loci.

Given that the CGH and FISH assays would miss some *MYC* rearrangements (e.g., balanced translocations; some inversions and insertions), it was estimated that *MYC* rearrangements are likely to have been present in ~60 % of the MMRC MM tumour samples [29]. Therefore, it seems that *MYC* is the most frequent target of genomic rearrangements in MM. It is clear that many *MYC* rearrangements occur during progression of MM. However, even though the increased expression of *MYC* in MM compared to MGUS often is mediated by a biallelic mechanism, it is possible that in some tumours, increased monoallelic expression of *MYC* mediated by *MYC* rearrangements is a driver of the MGUS to MM transition.

8.9 Complex *MYC* Rearrangements Mostly Hijack Nonrandom Super Enhancers

Enhancer DNA regulatory elements, which can directly regulate the transcriptional activity of genes, typically are located near or within the relevant gene(s), but sometimes can be located more than 1 Mb away from the gene [53, 54]. Enhancers can be identified by distinct patterns of histone marks (e.g., H3K27Ac), and the presence of specific transcription factors (e.g., *MED1* or *BRD4*) [55–57] (Table 8.2). Super enhancers (SE) typically are associated with genes that generally are expressed at a high level in specific cell types [55, 56]. Compared to conventional strong enhancers, SEs are identified by having a larger size and increased levels of *MED1*, *BRD4*, and H3K27Ac. Stretch enhancers (STRE), which can be weak or strong enhancers but have a size of at least 3 kb, often are tissue-specific and overlap locus control regions [57]. SEs have been identified in the MM.1S MMCL and the GM12878 lymphoblastoid cell line, which is phenotypically similar to MM.1S. STRE have been identified in GM12878 but not in MM.1S.

Table 8.2 Characteristics of enhancers juxtaposed to MYC in multiple myeloma

	Super (Loven)	Super (Hnisz)	Stretch (Parker)	Conventional
Marks	MED1, BRD4	H3K27Ac	6 ChIP marks[a]	–
Clustering	Stitched (<12.5 kb gaps)	Stitched	No	No
Median size	19 kb	8.7 kb	>3 kb (no gaps)	~1 kb
Number in MM.1S (MMCL)	308	640	ND	~10,000
Number in GM12878 (LCL)		257	10,615 (2318>6 kb)	10,000 (Hnisz) 100,000 (Parker)[b]
Rearranged near MYC >> MYCN > MYCL in MM	IGH, IGL, IGK, IGJ, TXNDC5, FAM46C, FOXO3, PRDM1, SNX5, ANKRD55, FAM188A	NSMCE2	MTSS1, AHNAK, TRIB1, PVT1	

[a]CTCF, H3K4me3, H3K4me, H3K27ac, H3K27me3, H3K36me3
[b]Parker data includes weak conventional enhancers

MYC:*IG* rearrangements in MMCL and MM tumours have been characterized by a combination of FISH, CGH, mate-pair sequences, cloned sequences, and whole genome sequencing [18, 20, 27, 29, 40, 58–60]. Although some of the *MYC*:*IG* rearrangements are simple reciprocal translocations, most of them are complex translocations or insertions (Fig. 8.1).

The major reason for complexity may be related to aberrant mechanisms that are responsible for karyotypic complexity in most kinds of tumours, including MM. However, in some cases the complexity seems to have a more direct role in the process of dysregulating *MYC*, a conclusion supported by three examples: (1) in the Karpas 620 MMCL, a der(14) t(11;14) with dysregulation of *CCND1* undergoes a rearrangement with chr8 that results in a der(8)t(8;14;11) and a der(14)t(8;14;11) that share ~1,700 kb of sequences from chromosomes 8,11, and 14, including *MYC* and the 3′*IGH* SEs [60]; (2) in the RPMI 8226 MMCL, the insertion of *MYC* at a der(16)t(16;22) breakpoint is associated with a duplication of the 3′*IGL* SE so that both *MAF* and *MYC* are dysregulated by different 3′E.*IGL* SE sequences (WMK&PLB, unpublished); and (3) in the LP1 MMCL, a variant *IGH* translocation, i.e. der(8) t(8;14), includes an inversion on chromosome 14 so that the insulator sequences that normally are centromeric to the 3′*IGH* SE are not positioned between the SE and *MYC*, which is located centromeric to the SE for this translocation [29].

Recently the first molecular details about non-*IG MYC* locus rearrangements in MMCLs or MM tumours have been reported [29, 60]. The structures of these rearrangements involving *MYC* (or *MYCL*) were deduced from a combination of FISH, CGH, mate-pair sequences, cloned sequences, and whole genome sequences of 14 MMCLs and 11 MMRC MM tumours. In 18 of these 25 non-*IG MYC* locus rearrangements, *MYC* is repositioned near a nonrandom group of MM.1S SEs (*NSMCE2* [8q24.13], *TXNDC5* [6p24.3], *FAM46C* [1p12], *FOXO3* [6q21], *PRDM1* [6q21], *IGJ* [4q13.3], *SNX5* [20p11.2], *ANKRD55* [5q11.2], *FAM188A* [10p13]) or GM12878 STREs (*AHNAK* [11q12.3], *MTSS1* [8q24.13], *TRIB1* [8q24.13]) (Table 8.2).

Several of the SEs were recurrent (*NSMCE2, TXNDC5, FAM46C, FOXO3, SNX5*). Moreover, samples with centromeric CNAs appear to be mostly deletions or inversions that reposition *MYC* relatively close to SEs in intron 4 of *NSMCE2*, which is ~2,500 kb upstream of *MYC*, or to STREs associated with *TRIB1*, which is ~2,300 kb upstream of *MYC*. Six of the other seven non-*IG MYC* locus rearrangements position *MYC* near conventional enhancers (two), centromeric to *NBEA* [13q13.3] but not close to any obvious enhancer (two), or with large tandem duplications that are located 350–500 kb downstream of *MYC* (two).

8.10 Characteristics of Secondary Immunoglobulin Rearrangements in Multiple Myeloma

Secondary rearrangements are not highly recurrent for partners other than *MYC* and, therefore, it has not been possible to compare the prevalence of these events in MGUS and MM. However, there are a few cases for which heterogeneity of a rearrangement in MM tumours suggests that a rearrangement is a secondary event. For example, the recently reported complex t(7;14) rearrangement, which is associated with ectopic expression of *EGFR*, was present in 85 % of MM tumour cells whereas other chromosome abnormalities were present in 97–99 % of tumour cells [61]. In the absence of more definitive proof, the following criteria, which are based on the analysis of *MYC* rearrangements, are suggestive that a rearrangement is secondary: (1) rearrangements are usually unbalanced translocations or insertions, often with involvement of three chromosomes, and sometimes with associated amplification, duplication, deletion, or inversion; and (2) rearrangement breakpoints usually are not within or very close to *IGH* switch regions or VDJ sequences even though these regions might sometimes be targeted by mechanisms other than the B cell specific DNA modification processes that are thought to be turned off in MGUS and MM tumours [18, 28]. Using these criteria, it is likely that rearrangements involving *IGK* and *IGL* are mostly secondary events, since they mostly are complex rearrangements that often involve *MYC* and rarely involve any of the seven primary *IGH* translocation partners [27]. Most *IGH* insertions and *IGH* rearrangements not involving one of the seven primary partners appear to be secondary events. However, the rare examples of MGUS or MM tumours with *IGH* rearrangements that target two of the primary partners suggest that one of the rearrangements is secondary [24, 27]; this is especially convincing for an MGUS tumour for which FISH identifies the t(4;14) in all tumour cells but the t(11;14) in ~30 % of tumour cells. In addition, *MAFB* expression is increased in the L363 MMCL, which has a complex rearrangement that positions *MAFB* near a *PDK1* SE (WMK&PLB, unpublished), and in the XG-2 MMCL, which has 3′ IGL sequences inserted near *MAFB* [27]. Therefore, it appears that *MAF* and *MAFB*, which are primary *IGH* translocation targets, may be particularly prone to be targets of secondary rearrangements. Secondary *IG* rearrangements have a similar prevalence in NHRD and HRD tumours, but *MYC* rearrangements not involving *IG* loci are significantly more frequent in HRD tumours than in NHRD tumours [20, 27, 29].

8.11 Concluding Thoughts and Unresolved Issues

Despite continued progress in our understanding of the roles of primary and secondary immunoglobulin rearrangements in the pathogenesis of MGUS and MM tumours, many important questions remain unanswered, some of which are briefly summarized below:

1. Are any of the primary translocations sufficient to cause MGUS? This does not seem to be the case for the t(11;14) translocations based on Eμ. CCND1 transgenic mice [45, 46] or for the t(4;14) since hybrid transcripts of *MMSET* are frequently found in healthy individuals (Marta Chesi, personal communication).
2. Primary *IGH* translocations target *CCND1>>CCND3>CCND2* both in MM and in mantle cell lymphoma despite the fact that B lineage cells express *CCND2* and *CCND3*, but little or no *CCND1*. There still is no compelling explanation for the more frequent involvement of *CCND1*.
3. Weinhold et al. [62] recently reported that the G allele for the rs603965 germline polymorphism(codon 870 G/A) is significantly associated (OR ~2, $P < 10^{-10}$) specifically with t(11;14) MGUS and MM but not t(11;14) mantle cell lymphoma. This is the first example of a specific translocation in MM that is associated with a germline polymorphism. It is unclear if the more efficient splicing of the G allele somehow enhances selection of cells with a t(11;14), or whether the G allele is more susceptible to acquiring a t(11;14).
4. Lopez-Corral et al. [63] have reported FISH studies showing that only a subclonal population of MGUS tumour cells derived from an individual patient have: t(4;14)[median 0.48]; t(11;14)[median 0.38]; or t(14;16)[0.30], with a higher fraction of smouldering MM having these translocations, and a still higher fraction having these translocations in symptomatic MM. They suggest that the t(4;14), t(11;14), and t(14;16) primary *IGH* translocations may actually be secondary events, with the tumour cell generated by the initial and unknown transforming events being outgrown by tumour cells that subsequently acquire one of these *IGH* translocations. This is a provocative result, which seems inconsistent with studies reported by others [20, 24, 64, 65] and, therefore, needs more rigorous validation of the hypothesis that they suggest.
5. Hebraud et al. [66] have reported that the t(4;14) can be present in only minor subclones of MM, and that there can be an apparent gain or loss of the t(4;14) during tumour progression. For most of the patients in this study, the observations were based on FISH studies that might not always detect the rearranged *MMSET* gene if the telomeric *IGH* sequences on the der(4) were lost during chromosome remodelling. In addition, it is unfortunate that the results reported in this study had very little experimental detail. In any case, this clearly is a provocative result that requires rigorous validation by other groups before their hypothesis can be accepted.
6. As indicated above, it remains to be rigorously confirmed that rearrangements mediated by apparent errors in D>J occur in pro-B cells and not at later stages of B cell development [43].

7. When do *MYC* rearrangements occur during pathogenesis? Interphase FISH studies suggest that they have a low prevalence (~3 %) in MGUS compared to MM (~16 %). Perhaps some of the MGUS tumours in that study were early MM or contained a combination of MGUS and MM cells. It is clear that some *MYC* rearrangements occur during progression of MM, but how often do *MYC* rearrangements occur in MGUS and drive the transition of MGUS to MM?
8. What is the full spectrum of super enhancers/stretch enhancers/conventional enhancers that are involved in *MYC* rearrangements? What is the basis for a nonrandom selection of these regulatory elements for *MYC* rearrangements?
9. Will the identification of recurrent super enhancers or stretch enhancers involved in *MYC* rearrangements lead to the identification of novel oncogenes that are dysregulated by juxtaposition to these regulatory elements in MM and other kinds of tumours?

References

1. Kuehl WM, Bergsagel PL (2012) Molecular pathogenesis of multiple myeloma and its premalignant precursor. J Clin Invest 122(10):3456–3463
2. Malpas JS, Bergsagel DE, Kyle R (eds) (2004) Myeloma: biology and management. Oxford University Press, Oxford
3. Kyle RA, Therneau TM, Rajkumar SV, Larson DR, Plevak MF, Offord JR et al (2006) Prevalence of monoclonal gammopathy of undetermined significance. N Engl J Med 354(13):1362–1369
4. Zingone A, Kuehl WM (2011) Pathogenesis of monoclonal gammopathy of undetermined significance and progression to multiple myeloma. Semin Hematol 48(1):4–12
5. Kyle RA, Durie BG, Rajkumar SV, Landgren O, Blade J, Merlini G et al (2010) Monoclonal gammopathy of undetermined significance (MGUS) and smoldering (asymptomatic) multiple myeloma: IMWG consensus perspectives risk factors for progression and guidelines for monitoring and management. Leukemia 24(6):1121–1127
6. Tiedemann RE, Gonzalez-Paz N, Kyle RA, Santana-Davila R, Price-Troska T, Van Wier SA et al (2008) Genetic aberrations and survival in plasma cell leukemia. Leukemia 22(5):1044–1052
7. Drexler HG, Matsuo Y (2000) Malignant hematopoietic cell lines: in vitro models for the study of multiple myeloma and plasma cell leukemia. Leuk Res 24(8):681–703
8. Kuehl WM, Bergsagel PL (2002) Multiple myeloma: evolving genetic events and host interactions. Nat Rev Cancer 2(3):175–187
9. Bakkus MH, Heirman C, Van Riet I, Van Camp B, Thielemans K (1992) Evidence that multiple myeloma Ig heavy chain VDJ genes contain somatic mutations but show no intraclonal variation. Blood 80(9):2326–2335
10. Dispenzieri A, Katzmann JA, Kyle RA, Larson DR, Melton LJ III, Colby CL et al (2010) Prevalence and risk of progression of light-chain monoclonal gammopathy of undetermined significance: a retrospective population-based cohort study. Lancet 375:1721–1728
11. Kuppers R (2005) Mechanisms of B-cell lymphoma pathogenesis. Nat Rev Cancer 5(4):251–262
12. Max EE, Fugman S (2013) Immunoglobulins: molecular genetics. In: Paul WE (ed) Fundamental immunology, 7th edn. Lippincott Williams & Wilkins, Philadelphia, pp 150–182
13. Bergsagel PL, Chesi M, Nardini E, Brents LA, Kirby SL, Kuehl WM (1996) Promiscuous translocations into immunoglobulin heavy chain switch regions in multiple myeloma. Proc Natl Acad Sci U S A 93(24):13931–13936

14. White MB, Word CJ, Humphries CG, Blattner FR, Tucker PW (1990) Immunoglobulin D switching can occur through homologous recombination in human B cells. Mol Cell Biol 10(7):3690–3699
15. Greisman HA, Lu Z, Tsai AG, Greiner TC, Yi HS, Lieber MR (2012) IgH partner breakpoint sequences provide evidence that AID initiates t(11;14) and t(8;14) chromosomal breaks in mantle cell and Burkitt lymphomas. Blood 120(14):2864–2867
16. Rossi D, Berra E, Cerri M, Deambrogi C, Barbieri C, Franceschetti S et al (2006) Aberrant somatic hypermutation in transformation of follicular lymphoma and chronic lymphocytic leukemia to diffuse large B-cell lymphoma. Haematologica 91(10):1405–1409
17. Chesi M, Nardini E, Lim RS, Smith KD, Kuehl WM, Bergsagel PL (1998) The t(4;14) translocation in myeloma dysregulates both FGFR3 and a novel gene, MMSET, resulting in IgH/MMSET hybrid transcripts. Blood 92(9):3025–3034
18. Bergsagel PL, Kuehl WM (2001) Chromosome translocations in multiple myeloma. Oncogene 20(40):5611–5622
19. Ahmann GJ, Jalal SM, Juneau AL, Christensen ER, Hanson CA, Dewald GW et al (1998) A novel three-color, clone-specific fluorescence in situ hybridization procedure for monoclonal gammopathies. Cancer Genet Cytogenet 101(1):7–11
20. Chiecchio L, Dagrada GP, Ibrahim AH, Dachs Cabanas E, Protheroe RK, Stockley DM et al (2009) Timing of acquisition of deletion 13 in plasma cell dyscrasias is dependent on genetic context. Haematologica 94(12):1708–1713
21. Kim GY, Gabrea A, Demchenko YN, Bergsagel L, Roschke AV, Kuehl WM (2014) Complex *IGH* rearrangements in multiple myeloma: frequent detection discrepancies among three different probe sets. Genes Chromosomes Cancer 53(6):467–474
22. Avet-Loiseau H, Daviet A, Brigaudeau C, Callet-Bauchu E, Terre C, Lafage-Pochitaloff M et al (2001) Cytogenetic, interphase, and multicolor fluorescence in situ hybridization analyses in primary plasma cell leukemia: a study of 40 patients at diagnosis, on behalf of the Intergroupe Francophone du Myelome and the Groupe Francais de Cytogenetique Hematologique. Blood 97(3):822–825
23. Avet-Loiseau H, Facon T, Grosbois B, Magrangeas F, Rapp MJ, Harousseau JL et al (2002) Oncogenesis of multiple myeloma: 14q32 and 13q chromosomal abnormalities are not randomly distributed, but correlate with natural history, immunological features, and clinical presentation. Blood 99(6):2185–2191
24. Fonseca R, Bailey RJ, Ahmann GJ, Rajkumar SV, Hoyer JD, Lust JA et al (2002) Genomic abnormalities in monoclonal gammopathy of undetermined significance. Blood 100(4):1417–1424
25. Fonseca R, Bergsagel PL, Drach J, Shaughnessy J, Gutierrez N, Stewart AK et al (2009) International Myeloma Working Group molecular classification of multiple myeloma: spotlight review. Leukemia 23(12):2210–2221
26. Fonseca R, Blood E, Rue M, Harrington D, Oken MM, Kyle RA et al (2003) Clinical and biologic implications of recurrent genomic aberrations in myeloma. Blood 101(11):4569–4575
27. Gabrea A, Martelli ML, Qi Y, Roschke A, Barlogie B, Shaughnessy JD Jr et al (2008) Secondary genomic rearrangements involving immunoglobulin or *MYC* loci show similar prevalences in hyperdiploid and nonhyperdiploid myeloma tumours. Genes Chromosomes Cancer 47(7):573–590
28. Gabrea A, Bergsagel PL, Michael Kuehl W (2006) Distinguishing primary and secondary translocations in multiple myeloma. DNA Repair 5(9-10):1225–1233
29. Affer M, Chesi M, Chen WD, Keats JJ, Demchenko YN, Tamizhmani K et al (2014) Promiscuous *MYC* locus rearrangements hijack enhancers but mostly super-enhancers to dysregulate *MYC* expression in multiple myeloma. Leukemia 28:1725–1735
30. Bergsagel PL, Kuehl WM, Zhan F, Sawyer J, Barlogie B, Shaughnessy J Jr (2005) Cyclin D dysregulation: an early and unifying pathogenic event in multiple myeloma. Blood 106(1):296–303

31. Zhan F, Huang Y, Colla S, Stewart JP, Hanamura I, Gupta S et al (2006) The molecular classification of multiple myeloma. Blood 108(6):2020–2028
32. Bochtler T, Hegenbart U, Cremer FW, Heiss C, Benner A, Hose D et al (2008) Evaluation of the cytogenetic aberration pattern in amyloid light chain amyloidosis as compared with monoclonal gammopathy of undetermined significance reveals common pathways of karyotypic instability. Blood 111(9):4700–4705
33. Hayman SR, Bailey RJ, Jalal SM, Ahmann GJ, Dispenzieri A, Gertz MA et al (2001) Translocations involving the immunoglobulin heavy-chain locus are possible early genetic events in patients with primary systemic amyloidosis. Blood 98(7):2266–2268
34. Chesi M, Bergsagel PL, Kuehl WM (2002) The enigma of ectopic expression of FGFR3 in multiple myeloma: a critical initiating event or just a target for mutational activation during tumor progression. Curr Opin Hematol 9(4):288–293
35. Keats JJ, Reiman T, Maxwell CA, Taylor BJ, Larratt LM, Mant MJ et al (2003) In multiple myeloma, t(4;14)(p16;q32) is an adverse prognostic factor irrespective of FGFR3 expression. Blood 101(4):1520–1529
36. Santra M, Zhan F, Tian E, Barlogie B, Shaughnessy J Jr (2003) A subset of multiple myeloma harboring the t(4;14)(p16;q32) translocation lacks FGFR3 expression but maintains an *IGH*/MMSET fusion transcript. Blood 101(6):2374–2376
37. Sawyer JR, Lukacs JL, Munshi N, Desikan KR, Singhal S, Mehta J et al (1998) Identification of new nonrandom translocations in multiple myeloma with multicolor spectral karyotyping. Blood 92(11):4269–4278
38. Sawyer JR, Lukacs JL, Thomas EL, Swanson CM, Goosen LS, Sammartino G et al (2001) Multicolour spectral karyotyping identifies new translocations and a recurring pathway for chromosome loss in multiple myeloma. Br J Haematol 112(1):167–174
39. Shaughnessy J Jr, Gabrea A, Qi Y, Brents L, Zhan F, Tian E et al (2001) Cyclin D3 at 6p21 is dysregulated by recurrent chromosomal translocations to immunoglobulin loci in multiple myeloma. Blood 98(1):217–223
40. Shou Y, Martelli ML, Gabrea A, Qi Y, Brents LA, Roschke A et al (2000) Diverse karyotypic abnormalities of the c-myc locus associated with c-myc dysregulation and tumor progression in multiple myeloma. Proc Natl Acad Sci U S A 97(1):228–233
41. Vatsveen TK, Tian E, Kresse SH, Meza-Zepeda LA, Gabrea A, Glebov O et al (2009) OH-2, a hyperdiploid myeloma cell line without an *IGH* translocation, has a complex translocation juxtaposing *MYC* near MAFB and the IGK locus. Leuk Res 33(12):1670–1677
42. Chapman MA, Lawrence MS, Keats JJ, Cibulskis K, Sougnez C, Schinzel AC et al (2011) Initial genome sequencing and analysis of multiple myeloma. Nature 471(7339):467–472
43. Walker BA, Wardell CP, Johnson DC, Kaiser MF, Begum DB, Dahir NB et al (2013) Characterization of *IGH* locus breakpoints in multiple myeloma indicates a subset of translocations appear to occur in pregerminal center B cells. Blood 121(17):3413–3419
44. Gabrea A, Bergsagel PL, Chesi M, Shou Y, Kuehl WM (1999) Insertion of excised IgH switch sequences causes overexpression of cyclin D1 in a myeloma tumour cell. Mol Cell 3(1):119–123
45. Bodrug SE, Warner BJ, Bath ML, Lindeman GJ, Harris AW, Adams JM (1994) Cyclin D1 transgene impedes lymphocyte maturation and collaborates in lymphomagenesis with the myc gene. EMBO J 13(9):2124–2130
46. Lovec H, Grzeschiczek A, Kowalski MB, Moroy T (1994) Cyclin D1/bcl-1 cooperates with myc genes in the generation of B-cell lymphoma in transgenic mice. EMBO J 13(15):3487–3495
47. Fonseca R, Debes-Marun CS, Picken EB, Dewald GW, Bryant SC, Winkler JM et al (2003) The recurrent IgH translocations are highly associated with nonhyperdiploid variant multiple myeloma. Blood 102(7):2562–2567
48. Smadja NV, Leroux D, Soulier J, Dumont S, Arnould C, Taviaux S et al (2003) Further cytogenetic characterization of multiple myeloma confirms that 14q32 translocations are a very rare event in hyperdiploid cases. Genes Chromosomes Cancer 38(3):234–239

49. Chng WJ, Van Wier SA, Ahmann GJ, Winkler JM, Jalal SM, Bergsagel PL et al (2005) A validated FISH trisomy index demonstrates the hyperdiploid and nonhyperdiploid dichotomy in MGUS. Blood 106(6):2156–2161
50. Ross FM, Ibrahim AH, Vilain-Holmes A, Winfield MO, Chiecchio L, Protheroe RK et al (2005) Age has a profound effect on the incidence and significance of chromosome abnormalities in myeloma. Leukemia 19(9):1634–1642
51. Avet-Loiseau H, Gerson F, Magrangeas F, Minvielle S, Harousseau JL, Bataille R (2001) Rearrangements of the c-myc oncogene are present in 15% of primary human multiple myeloma tumours. Blood 98(10):3082–3086
52. Chiecchio L, Dagrada GP, Protheroe RKM, Stockley DM, Smith AG, Orchard KH et al (2009) Loss of 1p rearrangements of MYC are associated with progression of smouldering myeloma to myeloma: sequential analysis of a single case. Haematologica 94(7):1024–1028
53. Nora EP, Dekker J, Heard E (2013) Segmental folding of chromosomes: a basis for structural and regulatory chromosomal neighborhoods? Bioessays 35(9):818–828
54. Shi J, Whyte WA, Zepeda-Mendoza CJ, Milazzo JP, Shen C, Roe JS et al (2013) Role of SWI/SNF in acute leukemia maintenance and enhancer-mediated Myc regulation. Genes Dev 27(24):2648–2662
55. Hnisz D, Abraham BJ, Lee TI, Lau A, Saint-Andre V, Sigova AA et al (2013) Super-enhancers in the control of cell identity and disease. Cell 155(4):934–947
56. Loven J, Hoke HA, Lin CY, Lau A, Orlando DA, Vakoc CR et al (2013) Selective inhibition of tumor oncogenes by disruption of super-enhancers. Cell 153(2):320–334
57. Parker SC, Stitzel ML, Taylor DL, Orozco JM, Erdos MR, Akiyama JA et al (2013) Chromatin stretch enhancer states drive cell-specific gene regulation and harbor human disease risk variants. Proc Natl Acad Sci U S A 110(44):17921–17926
58. Dib A, Gabrea A, Glebov OK, Bergsagel PL, Kuehl WM (2008) Characterization of *MYC* translocations in multiple myeloma cell lines. J Natl Cancer Inst 39:25–31
59. Yang L et al (2013) Diverse mechanisms of somatic structural variations in human cancer genomes. Cell 153(4):919–929
60. Walker BA, Wardell CP, Brioli A, Boyle E, Kaiser MF, Begum DB et al (2014) Translocations at 8q24 juxtapose MYC with genes that harbor superenhancers resulting in overexpression and poor prognosis in myeloma patients. Blood Cancer J 4, e191
61. Dib A, Glebov OK, Shou Y, Singer RH, Kuehl WM (2009) A der(8)t(8;11) chromosome in the Karpas-620 myeloma cell line expresses only cyclin D1: yet both cyclin D1 and MYC are repositioned in close proximity to the 3′IGH enhancer. DNA Repair 8(3):330–335
62. Walker BA, Wardell CP, Ross FM, Morgan GJ (2013) Identification of a novel t(7;14) translocation in multiple myeloma resulting in overexpression of EGFR. Genes Chromosomes Cancer 52(9):817–822
63. Weinhold N, Johnson DC, Chubb D, Chen B, Forsti A, Hosking FJ et al (2013) The CCND1 c.870G>A polymorphism is a risk factor for t(11;14)(q13;q32) multiple myeloma. Nat Genet 45(5):522–525
64. Lopez-Corral L, Gutierrez NC, Vidriales MB, Mateos MV, Rasillo A, Garcia-Sanz R et al (2011) The progression from MGUS to smoldering myeloma and eventually to multiple myeloma involves a clonal expansion of genetically abnormal plasma cells. Clin Cancer Res 17(7):1692–1700
65. Avet-Loiseau H, Facon T, Daviet A, Godon C, Rapp MJ, Harousseau JL et al (1999) 14q32 translocations and monosomy 13 observed in monoclonal gammopathy of undetermined significance delineate a multistep process for the oncogenesis of multiple myeloma. Intergroupe Francophone du Myelome. Cancer Res 59(18):4546–4550
66. Hebraud B, Caillot D, Corre J, Marit G, Hulin C, Leleu X et al (2013) The translocation t(4;14) can be present only in minor subclones in multiple myeloma. Clin Cancer Res 19(17):4634–4637

Chapter 9
Chromosomal Translocations in B Cell Lymphomas

Marco Fangazio, Laura Pasqualucci, and Riccardo Dalla-Favera

Contents

9.1 Introduction 158
9.2 Cell of Origin of B Cell Lymphomas 158
9.3 Mechanisms of Genetic Lesions in B Cell Lymphomas 160
9.3.1 Chromosomal Translocations 160
9.3.2 Gain-of-Function Mutations and Copy Number Gains 161
9.3.3 Deletions and Inactivating Mutations 164
9.3.4 Aberrant Somatic Hypermutation 164
9.4 Molecular Pathogenesis of Common B Cell Lymphomas 165
9.4.1 Mantle Cell Lymphoma 165
9.4.2 Burkitt Lymphoma 166
9.4.3 Follicular Lymphoma 169
9.4.4 Diffuse Large B Cell Lymphoma 170
9.4.4.1 Genetic Lesions Common to GCB- and ABC-DLBCL 170
9.4.4.2 Genetic Lesions of GCB-DLBCL 173
9.4.4.3 Genetic Lesions of ABC-DLBCL 173
9.4.4.4 Genetic Lesions of DLBCL Derived from CLL and FL Transformation 174
9.4.4.5 Genetic Lesions of PMBCL 176
References 176

Abstract B cell lymphomas represent a diverse group of biologically and clinically distinct neoplasms, encompassing over 40 subtypes that derive from the malignant transformation of mature B cells, most commonly at the germinal centre (GC) stage of differentiation. Analogous to most cancer types, these tumours are caused by alterations of oncogenes and tumour suppressor genes, some of which have

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specific roles in GC development. This chapter will focus on the mechanisms and consequences of chromosomal translocations and other genetic lesions involved in the pathogenesis of the most common types of mature B cell lymphomas, including Mantle Cell Lymphoma, Follicular Lymphoma, Diffuse Large B Cell Lymphoma, and Burkitt Lymphoma.

Keywords Germinal centre • Lymphoma • Genetic lesions • BCL6 • Immunoglobulin remodelling

9.1 Introduction

This chapter will focus on the role of chromosomal translocations and other mechanisms of genetic lesion in the pathogenesis of the most common and well-characterized types of B cell lymphoma (BCL), including Mantle Cell Lymphoma (MCL), Follicular Lymphoma (FL), Diffuse Large B Cell Lymphoma (DLBCL), and Burkitt Lymphoma (BL). Two additional common lymphoid malignancies, Chronic Lymphocytic Leukaemia (CLL) and Hodgkin Lymphoma (HL), will not be discussed in this chapter since either they lack recurrent chromosomal translocations (CLL) or their genome is still incompletely characterized (HL). Emphasis will be placed on the mechanisms of genetic lesions and the function of the involved genes in the context of normal B cell biology.

9.2 Cell of Origin of B Cell Lymphomas

Knowledge of the unique events that take place in the cell of origin of BCL is essential for understanding the mechanisms that are involved in the generation of chromosomal translocations and other BCL-associated genetic lesions. Most BCLs develop from the malignant expansion of mature B cells, and with the exception of MCL, arise from B cells that are arrested at various stages during their transit through a particular structure known as the germinal centre (GC). The GC is a specialized environment that forms in peripheral lymphoid organs when mature,

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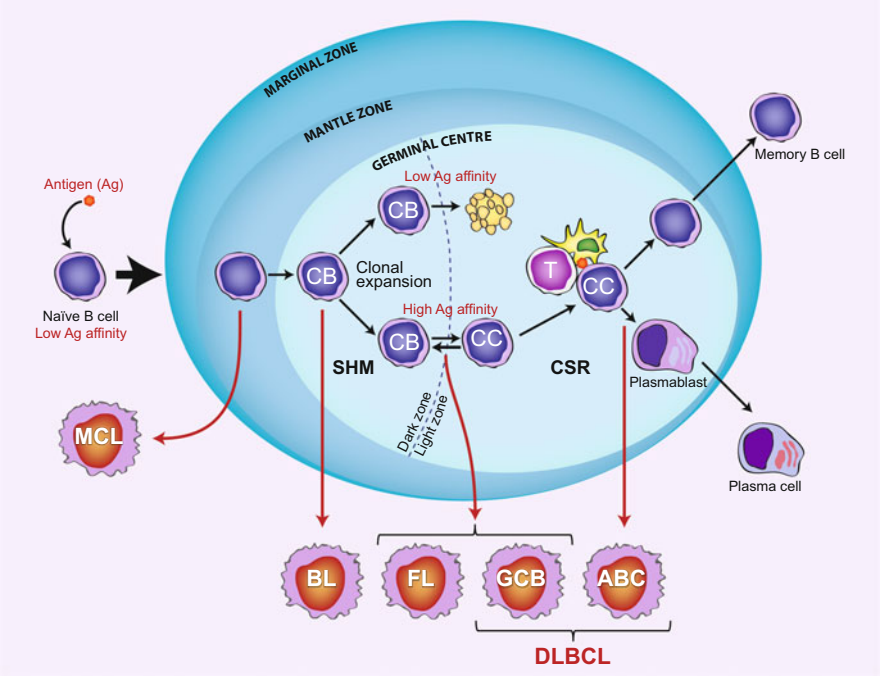

Fig. 9.1 The germinal centre reaction and lymphomagenesis. Schematic representation of a lymphoid follicle illustrating the germinal centre, the mantle zone, and the surrounding marginal zone. Upon encounter with a T-cell dependent antigen, naïve B cells undergo rapid proliferation and differentiate into centroblasts (CB) in the dark zone of the GC, where they also modify their *IG* genes by the process of SHM. CBs then transition into centrocytes (CC) in the light zone, where their encounter the antigen again, now presented by FDC, and, based on affinity for the antigen, are either selected to differentiate into plasma cells or memory B cells, re-enter the DZ, or be eliminated by apoptosis. In the light zone, CCs also undergo CSR. With the exception of mantle cell lymphoma (MCL), most BCL derive from cells that have experienced the GC reaction (*arrows*). FL, follicular lymphoma; BL, Burkitt lymphoma; DLBCL, diffuse large B cell lymphoma (*GCB* germinal centre B cell-like, *ABC* activated B cell-like)

naive B cells encounter a foreign antigen for the first time, in the context of signals delivered by CD4+ T cells and antigen-presenting cells (APC) (Fig. 9.1) [1–3].

GCs are characterized by two histologically and functionally well-defined zones: the dark zone (DZ), which consists of rapidly proliferating centroblasts (CBs) (doubling time 6–12 h), and the light zone (LZ), which is composed of more quiescent cells called centrocytes (CCs), surrounded by a network of follicular dendritic cells (FDC) and Tfh cells [4, 5]. In the DZ, the process of somatic hypermutation (SHM) modifies the variable region of the immunoglobulin (*IG*) genes, which encodes for the antigen-binding portion of the antibody, by introducing mostly point mutations that will change its affinity for the antigen [3, 6]. Following SHM in the DZ, CBs move to the LZ, where they compete for limited amount of antigen presented by FDCs. Based on the affinity of their B cell receptor (BCR) for the antigen, CCs will

then be selected to differentiate into memory B cells and plasma cells [3, 7] or, depending on stimulation by a variety of different signals, re-enter the DZ. In the GC, CCs also undergo class-switch recombination (CSR) [8], a DNA remodelling event that confers distinct effector functions to antibodies with identical specificities [9]. Both SHM and CSR represent B cell-specific functions that modify the genome of B cells via mechanisms involving single- or double-strand breaks, and both depend on the function of the activation-induced cytidine deaminase (AICDA/AID) enzyme [10, 11].

A master regulator of the GC reaction is the transcriptional repressor BCL6; this protein is specifically expressed in the GC and is an essential requirement for GC formation, as documented in vivo by mouse models where deletion of the *BCL6* gene was associated to the complete absence of these structures in response to antigenic stimulation [12–14]. BCL6 modulates the expression of numerous genes involved in BCR and CD40 signalling [15, 16], T-cell mediated B cell activation [15], apoptosis [15, 17], sensing and response to DNA damage [18–21], signalling pathways triggered by various cytokines and chemokines (e.g., interferon and TGFB1) [15, 17], and terminal B cell differentiation [22, 23]. BCL6 is therefore a central player in sustaining the proliferative nature of CBs, while allowing the execution of specific DNA remodelling processes (SHM and CSR) without eliciting responses to DNA damage. Furthermore, BCL6 suppresses a variety of signalling pathways that could lead to premature activation and differentiation before the selection of cells producing high-affinity antibodies. Once these processes are completed, multiple signals, including engagement of the BCR by the antigen and activation of the CD40 receptor by the CD40 ligand expressed on CD4+ T-cells, will induce the activation of different pathways and ultimately lead to downregulation of BCL6 at both the translational and transcriptional level, thus restoring the ability of the B cell to become activated and differentiate.

This simplified overview of the GC reaction is important to introduce two major concepts that are critical for the understanding of B cell lymphomagenesis: (i) as an irreversible marker of transit through the GC, the presence of somatically mutated *IG* genes in these tumours documented that the majority of BCLs, with the exception of most MCL cases, derive from the clonal expansion of GC-experienced B cells [24]; (ii) mistakes occurring during SHM and CSR are responsible for the generation of genetic alterations associated with BCL, including chromosomal translocations and aberrant somatic hypermutation (ASHM).

9.3 Mechanisms of Genetic Lesions in B Cell Lymphomas

9.3.1 Chromosomal Translocations

In B cell malignancies, chromosomal translocations occur at least in part as a consequence of mistakes in *IG* gene modification processes, and can thus be distinguished into three groups based on the structural features of the chromosomal breakpoint: (i) translocations due to errors occurring during the RAG-mediated

V(D)J recombination process (e.g. translocations involving *IGH* and *CCND1* in MCL [25] and translocations involving *IGH* and *BCL2* in FL) [26, 27]; (ii) translocations due to errors in the AICDA/AID -dependent CSR process (e.g., those involving the *IG* genes and *MYC* in sporadic BL) [26]; and (iii) translocations occurring as by-products of DNA breaks generated during the AICDA/AID-mediated SHM process (e.g., those joining the *IG* and *MYC* loci in endemic BL) [26]. Importantly, deletion of AICDA/AID in lymphoma-prone mouse models was shown to prevent both the occurrence of *IGH*/*MYC* translocations in normal B cells undergoing CSR [28, 29] and the development of GC-type lymphomas [30, 31], documenting the involvement and requirement of *IG* gene remodelling mechanisms in the pathogenesis of BCL.

In most chromosomal translocations associated with BCL, and in contrast with translocations associated with acute leukaemias, the coding domain of the involved proto-oncogene is left unaltered by the translocation, and no gene fusion is generated. Instead, heterologous regulatory sequences derived from the partner chromosome are juxtaposed in proximity of the oncogene, leading to deregulated expression of an intact protein. This process of proto-oncogene deregulation is defined as homotopic if a proto-oncogene whose expression is tightly regulated in the normal tumour counterpart becomes constitutively expressed in the lymphoma cell, and heterotopic if the proto-oncogene is not expressed in the putative normal counterpart of the tumour cell and undergoes ectopic expression in the lymphoma. In most types of BCL-associated translocations, the heterologous regulatory sequences responsible for proto-oncogene deregulation are derived from antigen receptor loci, which are expressed at high levels in the target tissue [26]. However, in certain translocations, such as the ones involving *BCL6* in DLBCL, different promoter regions from distinct chromosomal sites can be found juxtaposed to the proto-oncogene in individual tumour cases, a concept known as "promiscuous" translocations [32–40].

Only few BCL associated chromosomal translocations juxtapose the coding regions of the two involved genes, forming a chimeric transcriptional unit that encodes for a novel fusion protein, an outcome typically observed in chromosomal translocations associated with acute leukaemia. Examples include the t(11;18)(q22.2;q21.3) found in mucosa associated lymphoid tissue (MALT) lymphoma and the t(2;5)(p23.2;q35.1) typical of anaplastic large cell lymphoma (ALCL). The molecular cloning of the genetic loci involved in most recurrent translocations has led to the identification of a number of proto-oncogenes involved in lymphomagenesis (Supplemental Table 9.1).

9.3.2 Gain-of-Function Mutations and Copy Number Gains

The biological properties of a proto-oncogene can be altered by two additional mechanisms, including somatic point mutations and copy number gains/amplifications. Genomic mutations in the coding and/or regulatory region of a proto-oncogene may lead to stabilization or constitutive activation of its protein product. CN gains and

Supplemental Table 9.1 Most common chromosomal translocations associated with major B-NHL types

B-NHL type	Chromosomal translocation	Frequency	Involved gene	Functional consequences	Postulated mechanism of transformation
MCL	t(11;14)(q13.3;q32.3)	>95 %	*CCND1*	Deregulated expression	Enhanced proliferation and growth
BL	t(8;14)(q24.2;q32.3)	80 %	*MYC*	Deregulated expression	Enhanced proliferation and growth, aberrant DNA replication
	t(2;8)(p12;q24.2)	15 %	*MYC*	”	”
	t(8;22)(q24.2;q11.2)	5 %	*MYC*	”	”
FL	t(14;18)(q32.3;q21.3)	85 %	*BCL2*	Deregulated expression	Resistance to apoptosis
	t(2;18)(p12;q21.3)	rare	*BCL2*	”	”
	t(18;22)(q21.3;q11.2)	rare	*BCL2*	”	”
	t(3;various)(q27;various)	6–14 %	*BCL6*	”	Enhanced proliferation, impaired DNA damage responses, block of differentiation
MZL (MALT type)	t(11;18)(q22.2;q21.3)	60 %	*BIRC3*; *MALT1*	Fusion protein/transcriptional activation of BIRC3	Constitutive activation of the NF-κB signalling pathway
	t(1;14)(p22.3;q32.3)	4–9 %[a]	*BCL10*	Deregulated expression	Constitutive activation of the NF-κB signalling pathway
	t(14;18)(q32.3;q21.3)	7–38 %[a]	*MALT1*	Deregulated expression	Constitutive activation of the NF-κB signalling pathway
	t(3;14)(p13;q32.3)	5–20 %[a]	*FOXP1*	Deregulated expression	Unclear (transcription factor)

DLBCL, GCB-type	t(8;14)(q24.2;q32.3)	10 %	*MYC*	Deregulated expression	Enhanced proliferation and growth, aberrant DNA replication
	t(14;18)(q32.3;q21.3)	30–40 %	*BCL2*	”	Resistance to apoptosis
	t(3;various) (q27;various)	15 %	*BCL6*	”	Enhanced proliferation, impaired DNA damage responses, block of differentiation
DLBCL, ABC-type	t(3;various) (q27;various)	25 %	*BCL6*	Deregulated expression	Enhanced proliferation, impaired DNA damage responses, block of differentiation
PMBCL	t(16; various) (p13; various)	38 %	*CIITA*	Disruption of CIITA function	Reduced tumour cell immunogenicity, downregulation of HLA class II protein
LPL	t(9;14)(p13.2;q32.3)	50 %	*PAX5*	Deregulated expression	Altered B-cell proliferation and differentiation

LPL lymphoplasmacytic lymphoma
[a]Depending on the involved site

amplifications typically result in the overexpression of an intact protein. Over the past few years, the use of next-generation sequencing technologies and high density genomic arrays have led to the identification of numerous recurrent targets of somatic mutations and CN changes that likely play central roles in transformation. These genes will be discussed in individual disease sections. Of note, point mutations of the *RAS* genes, a very frequent proto-oncogene alteration in human neoplasia, are rare in lymphomas [41]. Also, only a few genes have been identified so far as specific targets of amplification in BCLs, including *REL* and *BCL2* in DLBCL [42–45] and the genes encoding for the PD ligands in primary mediastinal B cell lymphoma (PMBCL)[46].

9.3.3 Deletions and Inactivating Mutations

Recent genomic efforts have uncovered several new candidate tumour suppressor genes that are lost in BCLs due to chromosomal deletions and/or deleterious mutations. Among these genes, *PRDM1* (also known as *BLIMP1*) on 6q21 is biallelically inactivated in ~25 % of ABC-DLBCL cases [47–49]; and *TNFAIP3*, the gene encoding for the negative NF-κB-regulator A20 on 6q23, is inactivated in ~30 % of ABC-DLBCL, as well as in PMBCL, marginal-zone lymphoma and HL [50–53]. Heterozygous mutations and deletions inactivating the acetyltransferase genes *CREBBP* and *EP300* are observed in a significant fraction of DLBCL and FL, supporting a haploinsufficient tumour suppressor role [54]. DLBCL and FL also carry loss-of-function mutations of *KMT2D*/*MLL2*, a gene encoding for a methyltransferase found mutated in multiple cancer types [55, 56]. More than half of all CLL cases are associated with CN losses encompassing the *DLEU2*/*miR15-a*/*16.1* cluster on 13q14.3 [57–59], while the *CDKN2A*/*CDKN2B* locus is targeted by focal homozygous deletions in a large proportion of transformed FL (tFL), Richter syndrome (RS) and ABC-DLBCL cases [60–62], and is epigenetically silenced in various MCL cases [63]. Loss of the *TP53* tumour suppressor gene, likely the most commonly mutated gene in human cancer [64], is observed at relatively low frequencies in BCL, where these lesions seem preferentially associated with specific disease subtypes, including BL and DLBCL derived from the transformation of FL or CLL [65, 66]. Analogous to other neoplasms, the mechanism of *TP53* inactivation in BCL entails point mutation of one allele and chromosomal deletion or mutation of the second allele.

9.3.4 Aberrant Somatic Hypermutation

In normal GC B cells, the process of SHM is tightly regulated, introducing mutations only in the rearranged *IG* variable sequences [67] as well as in the 5′ region of a few other loci, including *BCL6* and the *CD79* components of the B cell receptor

[68–70], although the functional role of mutations found in non-*IG* genes remains obscure. On the contrary, multiple mutational events have been found to affect numerous loci in over half of DLBCL cases [71] and, at lower frequencies, in other lymphoma types [72–76], as the result of a pathologic phenomenon called aberrant somatic hypermutation (ASHM). These mutations are typically distributed within ~2 Kb from the transcription initiation site [77] and, depending on the genomic configuration of the target gene, may affect both coding and non-coding regions, thus holding the potential to alter the function of the encoded protein and its transcriptional regulation. The target loci identified to date include several well-known proto-oncogenes, such as *PIM1*, *PAX5* and *MYC* [71]. However, the mechanism underlying ASHM and a comprehensive genome-wide characterization of its consequences are still incompletely defined.

9.4 Molecular Pathogenesis of Common B Cell Lymphomas

9.4.1 Mantle Cell Lymphoma

Mantle cell lymphoma is a tumour of mature B cells expressing specific differentiation markers and characterized in most cases by unmutated *IGH* variable sequences, consistent with the derivation from naive, pre-GC peripheral B cells (Fig. 9.1). However, recent studies revealed the existence of cases that carry SHM-associated mutations (15–40 % of diagnoses), reflecting the influence of the GC environment.

MCL is characteristically associated with the t(11;14)(q13.3;q32.3) translocation, which juxtaposes the *IGH* gene to chromosomal region 11q13.3, containing the *CCND1* gene [25, 78, 79]. The translocation causes the heterotopic deregulation of cyclin D1, a member of the D-type G_1 cyclins that regulates the early phases of the cell cycle and is normally not expressed in resting B cells [80–82]. Another ~10 % of MCL patients over-express aberrant or shorter cyclin D1 transcripts resulting from secondary rearrangements, microdeletions or point mutations in the gene 3′ untranslated region [78, 83–85]. The tumourigenic role of cyclin D1 deregulation in human neoplasia is suggested by the ability of the overexpressed protein to transform cells in vitro and to induce B cell lymphomas in transgenic mice, although only when combined to other oncogenic alterations [86, 87]. Because of the elevated frequency and specificity of alterations, the ectopic expression of cyclin D1 in the tumour cells constitutes a standard immunohistochemical marker for MCL diagnosis [88].

Additional genetic alterations accompanying the t(11;14)(q13.3;q32.3) in MCL include deletions and mutations inactivating the *ATM* gene (~40 % of patients) [89], loss of *TP53* (20 %) [90], and inactivation of the *CDKN2A* gene by deletions, point mutations or promoter hypermethylation, more frequently observed in aggressive cases (67 %) [91]. Aggressive tumours are associated with mutations in *NOTCH1* (12 % of clinical samples) and *NOTCH2* (5 % of samples), which are mutually exclusive and are typically represented by frameshift or nonsense events leading to the loss

of the PEST sequences required for protein degradation and thus to stabilization of the NOTCH protein [92, 93]. Less common, yet recurrent and therefore presumably functionally relevant mutations involve *BIRC3*, the Toll-like receptor 2 (TLR2), the chromatin modifiers WHSC1 and KMT2D/MLL2, and the MEF2B transcription factor [92]. Finally, in a small number of cases, *BMI1* is amplified and/or overexpressed, possibly as an alternative mechanism to the loss of the *CDKN2A* cell cycle regulator gene [94].

9.4.2 Burkitt Lymphoma

BL derives from GC B cells displaying phenotypic and molecular features of transformed centroblasts, as documented by the presence of highly mutated *IG* variable sequences [95–97] and the expression of a distinct transcriptional signature [98, 99]. BL includes three clinical variants: sporadic BL (sBL), endemic BL (eBL) and HIV-associated BL, which is often diagnosed as a manifestation of AIDS [88].

The genetic hallmark of BL is a chromosomal translocation involving the *MYC* gene on chromosome 8q24.2 and one of the *IG* loci on the partner chromosome [100, 101], with *IGH* (14q32.3) being the most frequently involved (80 % of cases) and *IGK* (2p12) or *IGL* (22q11.2) being found in the remaining 20 % of cases [100–103]. These translocations show a high degree of molecular heterogeneity, since the breakpoints are located 5′ and centromeric to *MYC* in t(8;14), but map 3′ to *MYC* in t(2;8) and t(8;22) [100–104]. Further molecular heterogeneity derives from the breakpoint sites observed on chromosomes 8 and 14 in t(8;14): translocations of eBL tend to involve sequences at an undefined distance (>100 kb) 5′ to *MYC* on chromosome 8 and sequences within or in proximity to the Ig J_H region on chromosome 14 (Fig. 9.2) [105, 106]. In sBL, t(8;14) preferentially involves sequences within or immediately 5′ to *MYC* (<3 kb) on chromosome 8 and within the Ig switch regions on chromosome 14 (Fig. 9.2) [105, 106].

The different molecular architecture of these translocations is thought to reflect distinct mechanisms of *IG* gene remodelling involved in their generation, namely CSR in sBL and AIDS-BL and SHM in eBL [26].

All t(8;14), t(2;8) and t(8;22) lead to the ectopic expression of the MYC proto-oncogene [107–109], which is normally absent in the majority of proliferating GC B cells [1], where it is repressed by BCL6 [110]. Oncogenic activation of *MYC* in BL is mediated by at least three distinct mechanisms: (i) juxtaposition of the MYC coding sequences to heterologous enhancers derived from the *IG* loci [107–109]; (ii) point mutations in the gene 5′ regulatory sequences, which alter the responsiveness to cellular factors controlling its expression [111]; (iii) amino acid substitutions within the gene exon 2, encoding for the protein transactivation domain [112, 113]; these mutations can abolish the ability of RBL1/p107, a nuclear protein related to *RB1*, to suppress *MYC* activity [114], or can increase protein stability [115, 116].

MYC is a nuclear phosphoprotein that binds and transcriptionally regulates thousand of target genes with diverse roles in regulating cell growth by affecting DNA replication, energy metabolism, protein synthesis, and telomere elongation

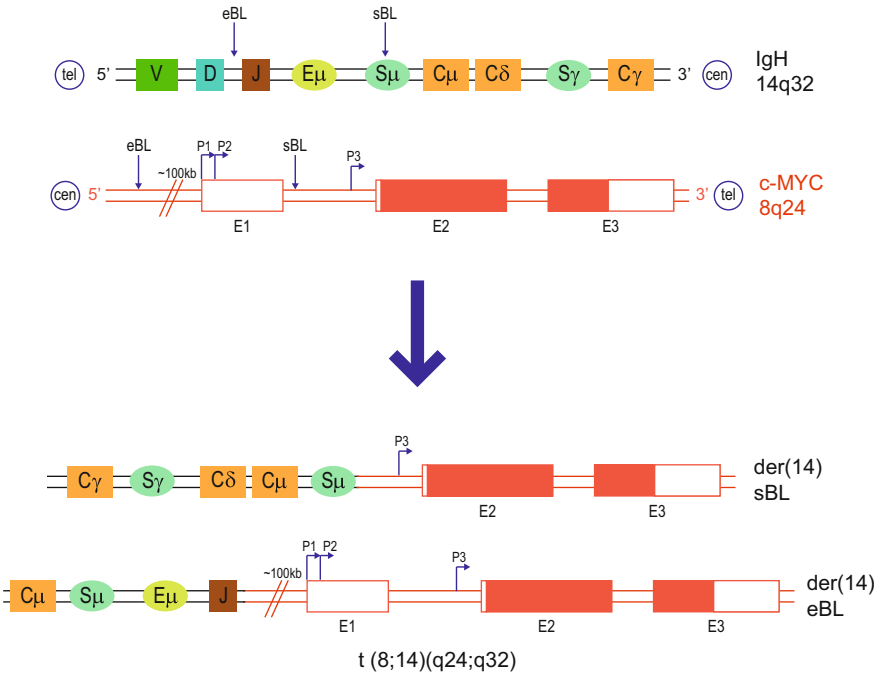

Fig. 9.2 Molecular anatomy of chromosomal translocations involving *MYC*. The *top panel* shows the genomic configuration of the germline *IGH* and *MYC* loci involved in the t(8;14) translocation (not in scale). Upon recombination, the *IGH* enhancer sequences are juxtaposed to the *MYC* coding region (*bottom panel*), causing deregulated expression of its protein product. Only one of the derivative chromosomes is shown. *TEL* telomeric end, *CEN* centromeric end

[117–119]. The deregulated expression of these functions is typically involved in malignant transformation. In addition, deregulated *MYC* expression is thought to cause genomic instability and, thus, contribute to tumour progression by facilitating the occurrence of additional genetic lesions [120]. Several transgenic mouse models of deregulated MYC expression have been generated and shown to develop aggressive B cell lymphomas with high penetrance and short latency [116, 121, 122]. In particular, the combination of deregulated expression of *MYC* and PI3K signalling activation in GC B cells leads to lymphomas recapitulating the features of human BL [123].

Genome sequencing has recently revealed additional oncogenic mechanisms that cooperate with MYC in the development of BL. Mutations affecting the genes encoding for the TCF3 transcription factor and for its negative regulator ID3 are frequently observed in all BL subtypes (10–25 % and 35–38 % of cases, respectively). These mutations trigger tonic (antigen-independent) BCR signalling and promote cell survival through activation of the PI3K signalling pathway (Fig. 9.3) [124].

TCF3 can also transactivate *CCND3*, promoting cell-cycle progression, while in 38 % of sBL, mutations within the carboxyl terminus domain of CCND3 stabilize the protein leading to higher expression levels. Other recurrent alterations associated

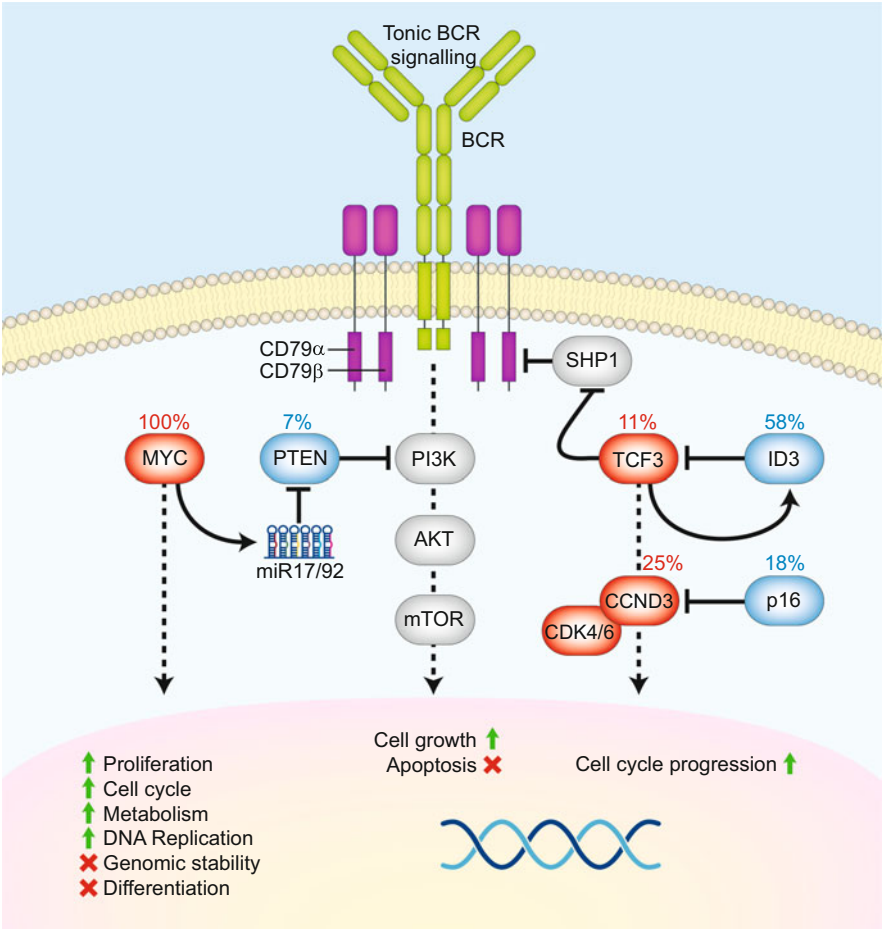

Fig. 9.3 Molecular basis of BL pathogenesis. Pathways affected by genetic aberrations in BL. Proteins in red are encoded by genes targeted by translocations (*MYC*) or activating mutations, and Proteins in blue denote genes targeted by inactivating events

with BL include *TP53* loss by mutation and/or deletion (35 % of both sBL and eBL cases) [65], *CDKN2B* inactivation by deletion or hypermethylation (17 % of samples) [125], and 6q deletions (~30 % of cases, independent of the clinical variant) [126]. Finally, one contributing factor to the development of BL is monoclonal EBV infection, present in virtually all cases of eBL and in ~30 % of sBL and AIDS-BL [127–130]. However, BL cells lack the expression of both EBV transforming antigens (LMP1 and EBNA2); considering also that this virus is endemic in humans worldwide, these observations raise some doubts on the pathogenic role of EBV in this disease [131].

9.4.3 *Follicular Lymphoma*

FL is characterized by an indolent clinical course but remains incurable and ultimately leads to death often accompanied by histologic transformation to an aggressive lymphoma with a DLBCL phenotype (20–30 % of cases) [132, 133]. The derivation of FL from a GC B cell is supported by the expression of specific GC B cell markers together with the presence of SHM-mutated *IG* genes [24].

Eighty to ninety percent of FL cases are characterized by chromosomal translocations that affect the *IG* locus and the *BCL2* gene on chromosome band 18q21.3 [78, 134–137]. These rearrangements join the 3′ untranslated region of *BCL2* to an *IG* J_H segment, leading to ectopic expression of the BCL2 protein in GC B cells [134, 135, 138–142], where its transcription is normally repressed by BCL6 [17, 143]. Approximately 70 % of the breakpoints on chromosome 18 cluster within the major breakpoint region, while the remaining 5–25 % map to the more distant minor cluster region, located ~20 kb downstream of the *BCL2* gene (Fig. 9.4)[134, 135, 138, 139]. More rarely, rearrangements involve the 5′ flanking sequences of *BCL2* (Fig. 9.4)[144]. *BCL2* encodes for a major negative regulator of programmed cell death and may thus contribute to lymphomagenesis by conferring resistance to

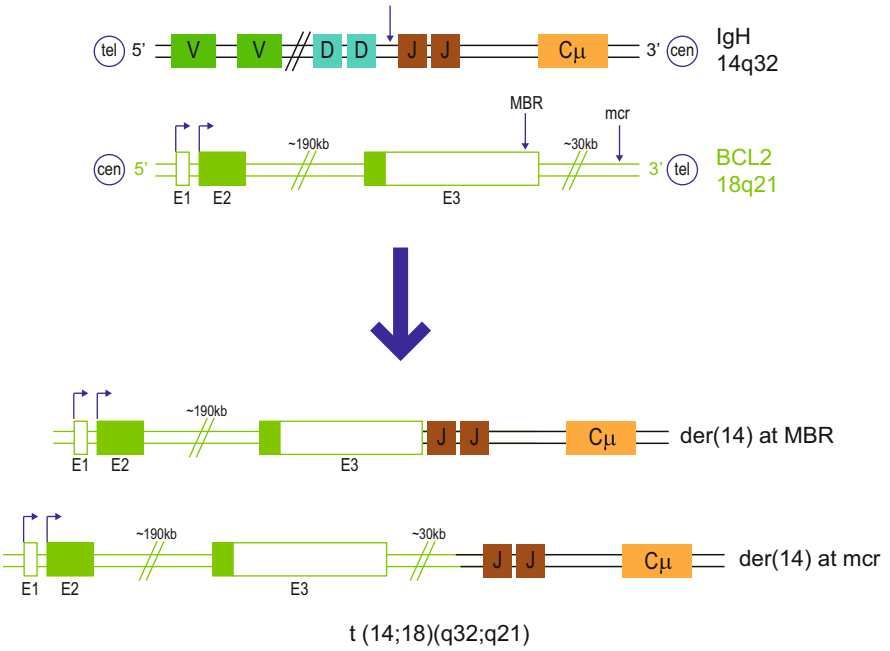

Fig. 9.4 Molecular anatomy of chromosomal translocations involving *BCL2*. *Top panel*, genomic configuration of the germline *IGH* and *BCL2* loci (not in scale). The translocation t(14;18) leads to the juxtaposition of the *IGH* enhancer sequences to the *BCL2* coding region (*bottom panel*), causing the deregulated expression of an intact protein. Only one of the derivative chromosomes is shown. *TEL* telomeric end, *CEN* centromeric end, *MBR* major breakpoint region, *mcr* minor cluster region

apoptosis independent of antigen selection. Other genes recurrently targeted by mutations in FL include those encoding for the methyltransferase KMT2D/MLL2 (up to 80 % of cases), the polycomb-group oncogene *EZH2* (7–20 % of patients), and the acetyltransferases *CREBBP* and *EP300* (40 % of cases), all of which may facilitate transformation by epigenetic remodelling of the precursor cancer cell.

The genomic analysis of clonally related FL and tFL biopsies has recently allowed the identification of the genetic lesions that are specifically acquired during histologic progression to DLBCL. These lesions include inactivation of *CDKN2A*/*CDKN2B* through deletion, mutation and hypermethylation (one third of patients) [61, 91], rearrangements and amplifications of *MYC* [145], *TP53* mutations/deletions (25–30 % of cases) [66, 146–148], loss of chromosome 6 (20 %) [126], and ASHM [61]. Additionally, Biallelic inactivation of the gene encoding *B2M*, leading to the loss of HLA class I expression on the cell surface of the tumour cells (see below) suggests that escape from immune surveillance may be important for FL transformation to DLBCL.

9.4.4 Diffuse Large B Cell Lymphoma

DLBCL is an aggressive disease that includes cases arising *de novo* as well as cases derived from the clinical evolution of FL and CLL [88]. Gene expression profile analysis has identified three well-characterized molecular subtypes of DLBCL, which reflect the derivation from different stages of B cell development. Germinal centre B cell-like (GCB) DLBCL is thought to derive from GC B cells with a phenotype intermediate between CB and CC; activated B cell-like (ABC) DLBCL is related to B cells committed to plasmablastic differentiation; and PMBCL arises from thymic B cells that have experienced the GC; the remaining 15–30 % of cases is still unclassified [149–153]. Of note, patients diagnosed with GCB-DLBCL have favourable prognosis compared to ABC-DLBCL [45].

Compared to other B cell malignancies, DLBCL shows a significantly higher degree of genomic complexity, carrying on average 50–100 lesions/case, with significant heterogeneity across patients [55, 56, 154]. Many of the lesions identified can be variably found in both molecular subtypes of the disease, consistent with a general role during transformation, while others appear to be preferentially or exclusively associated with individual DLBCL subtypes, indicating that GCB-DLBCL, ABC-DLBCL and PMBCL are genetically, phenotypically and clinically distinct diseases (Fig. 9.5).

9.4.4.1 Genetic Lesions Common to GCB- and ABC-DLBCL

A major contributor to DLBCL pathogenesis, in both GCB- and ABC-DLBCL, is represented by the deregulated activity of the BCL6 oncoprotein, which results from multiple genetic lesions. Chromosomal translocations involving the *BCL6* gene at band 3q27 are observed in up to 35 % of cases [155–157], with a twofold

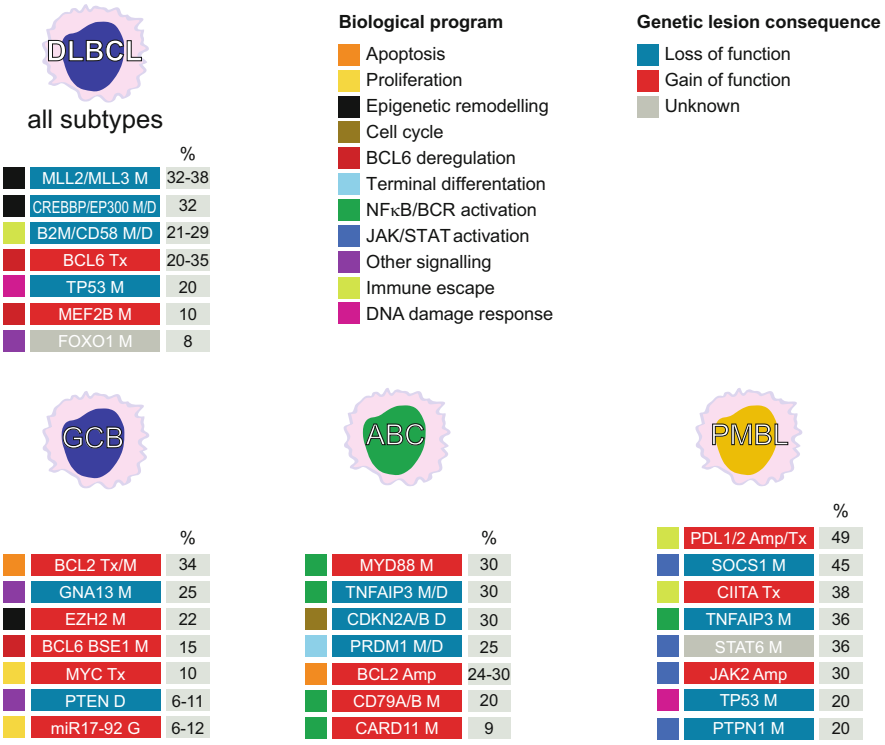

Fig. 9.5 Genetic lesions associated with DLBCL. Most common genetic alterations identified in GCB-DLBCL, ABC-DLBCL and PMBCL. The biological function/signalling pathway affected by the lesion is indicated by colour-coded squares and is explained in the *upper right panel*. *M* mutation, *D* deletion, *Tx* translocation, *Amp* amplification

higher frequency in the ABC-DLBCL subtype [158]. These translocations juxtapose the coding exons of *BCL6* downstream and in the same transcriptional orientation to heterologous sequences derived from a variety of partner chromosomes, including *IGH* (14q32.3), *IGK* (2p11.2), *IGL* (22q11.2), and at least 20 other chromosomal sites unrelated to the *IG* loci (Fig. 9.6) [32–39].

Most translocations result in a fusion transcript in which the promoter region and the first non-coding exon of *BCL6* are replaced by sequences derived from the partner gene [33, 159]. Since the common denominator of these promoters is the expression in the post-GC differentiation stage, the translocation is thought to prevent the downregulation of *BCL6* expression that is normally associated with differentiation into post-GC cells. Deregulated expression of an intact BCL6 gene product is also sustained by a variety of indirect mechanisms, including gain-of-function mutations in its positive regulator MEF2B (~11 % of cases) [160], inactivating mutations/deletions of *CREBBP*/*EP300* [54], which in normal cells impair BCL6 activity (see below) [161], and mutations/deletions of *FBXO11* (~5 %) [162], encoding a ubiquitin ligase involved in the control of BCL6 protein degradation. As documented by a mouse model in which deregulated BCL6 expression in GC B cells leads to the development

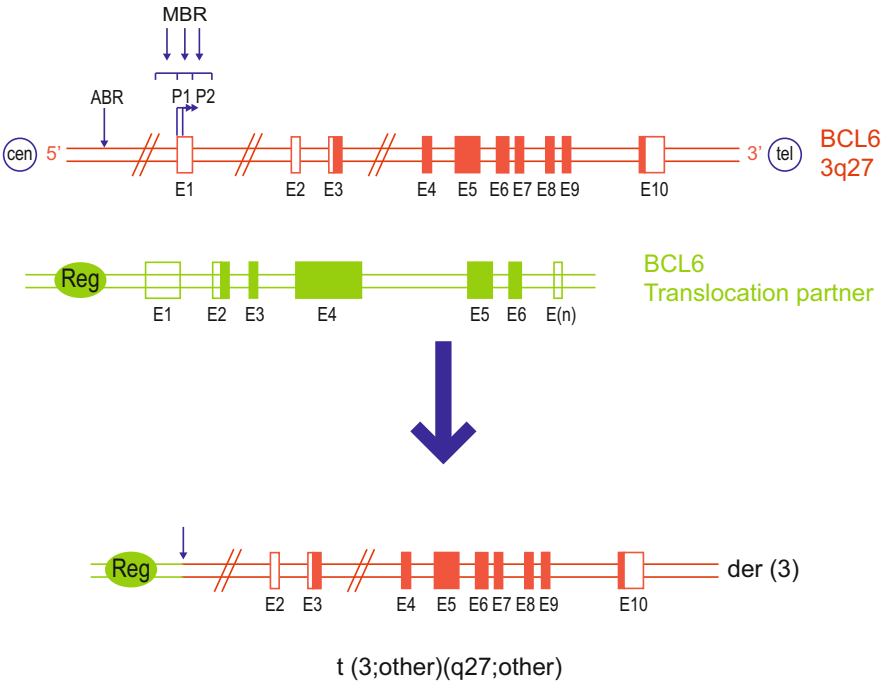

Fig. 9.6 Molecular anatomy of chromosomal translocations involving *BCL6*. *Top panel*, genomic configuration of the germline *BCL6* locus and a representative gene involved in the translocation partner chromosome (not in scale). *Bottom panel*, schematic representation of the derivative chromosome resulting from the translocation; only one of the derivative chromosomes is shown. *TEL* telomeric end, *CEN* centromeric end, *Reg* regulatory sequences

of DLBCL [163], BCL6 plays a critical role in lymphomagenesis by enforcing the proliferative phenotype typical of GC cells, suppressing proper DNA damage responses, and blocking terminal differentiation.

The most frequently disrupted function in DLBCL, independent of subtype, is represented by epigenetic remodelling, due to mutations in the *CREBBP/EP300* acetyltransferase genes (35 % of cases) [54] and the *KMT2D/MLL2* H3K4 methyltransferase (~30 % of all DLBCL) [54–56]. These lesions may favour malignant transformation by reprogramming the cancer epigenome, and in the case of *CREBBP/EP300*, by altering the balance between the activity of the *BCL6* oncogene, which is typically inactivated by acetylation, and the tumour suppressor TP53, which requires acetylation at specific residues for its function as a tumour suppressor [54].

Escape from both arms of immune surveillance, including CTL-mediated cytotoxicity (through genetic loss of the *B2M/HLA-I* genes) and NK cell-mediated death (through genetic loss of the CD58 molecule) also appears a major feature of the DLBCL phenotype [164]. Analogous effects may be achieved in PMBCL by

disruption of the MHC-II transactivator CIITA [165] and amplification of the genes encoding for the immunomodulatory proteins PDL1/PDL2 [46].

Finally, approximately 50 % of all DLBCL are associated with ASHM [71]. The number and identity of the genes that accumulate mutations in their coding and non-coding regions due to this mechanism varies in different cases and is still largely undefined [166]. ASHM may therefore contribute to the heterogeneity of DLBCL via the alteration of different cellular pathways in different cases.

9.4.4.2 Genetic Lesions of GCB-DLBCL

These include the t(14;18) and t(8;14) translocations, which deregulate the *BCL2* and *MYC* oncogenes in 34 % and 10 % of cases, respectively [45, 143, 167–169]. Virtually restricted to this subtype are also mutations of EZH2 [170], a histone methyltransferase that trimethylates Lys27 of histone H3 (H3K27); mutations of several genes in the Galpha13 pathway, including the *GNA13* gene, which are involved in the ability of DLBCL cells to spread from their lymphoid sites to the peripheral blood and bone marrow; and deletions of the tumour suppressor *PTEN* [62, 171]. Mutations affecting an autoregulatory domain within the *BCL6* 5′ untranslated exon 1 [158, 172, 173] are detected in up to 75 % of DLBCL cases [69, 174, 175], and reflect the activity of the physiologic SHM mechanism that operates in normal GC B cells [69, 176]. Functional analysis of numerous mutated *BCL6* alleles uncovered a subset of mutations that are specifically associated with GCB-DLBCL [172], and deregulate *BCL6* transcription by disrupting an autoregulatory circuit through which the BCL6 protein controls its own expression levels via binding to the promoter region of the gene [172, 173] or by preventing CD40-induced BCL6 downregulation in post-GC B cells [177]. However, the full extent of mutations deregulating BCL6 expression has not been characterized, and therefore the fraction of DLBCL cases carrying abnormalities in the *BCL6* gene remains undefined.

9.4.4.3 Genetic Lesions of ABC-DLBCL

ABC-DLBCL depends on the constitutive activation of the NF-κB signalling pathway caused by a variety of alterations in positive and negative regulators of NF-κB. In ~30 % of cases, the *TNFAIP3* gene, encoding for the negative regulator A20, is biallelically inactivated by mutations and/or deletions, thus preventing termination of NF-κB-responses [50, 51]. In an additional ~10 % of ABC-DLBCL, the *CARD11* gene is targeted by oncogenic mutations clustering in the protein coiled-coil domain and enhancing its ability to transactivate NF-κB-target genes [178]. Finally, nearly 30 % of ABC-DLBCL cases recurrently show a hotspot mutation (L265P) in the intracellular Toll/interleukin-1 receptor domain of the MYD88 adaptor molecule, which has the potential to activate NF-κB as well as JAK/STAT3 transcriptional responses [179]. At lower frequencies, mutations were found in a number of additional genes encoding for NF-κB pathway components.

Overall, lesions affecting NF-κB activation account for over 50 % of all ABC-DLBCL [50, 51], suggesting that additional mechanisms and/or yet unidentified lesions are responsible for the constitutive NF-κB activity in the remaining cases.

ABC-DLBCLs also depend upon chronic active BCR signalling (which also lead to NF-κB activation). This is associated in ~10 % of cases with somatic mutations of *CD79B* and *CD79A* [180], typically located within the immunoreceptor tyrosine-based activation motif (ITAM). Since silencing of several BCR proximal and distal subunits is toxic to ABC-DLBCL [180], there is conceptual support for the development of therapies that target BCR signalling components. In fact, preliminary data suggest that the Bruton Tyrosine Kinase (BTK) inhibitor Ibrutinib, may be effective against a subset of ABC-DLBCL cases.

Biallelic truncating or missense mutations and/or genomic deletions of the *PRDM1*/*BLIMP1* gene, which encodes for a zinc finger transcriptional repressor required for terminal B cell differentiation [181], block DLBCL cells in the plasmablastic stage in ~25 % of ABC-DLBCL [47–49]. In an additional 25 % of cases, the same consequence is caused by transcriptional repression of PRDM1/BLIMP1 by constitutively active BCL6 alleles [47–49]. Accordingly, translocations deregulating the *BCL6* gene and *BLIMP1* inactivation are mutually exclusive in DLBCL, consistent with these alterations representing alternative oncogenic mechanisms contributing to blocking differentiation during lymphomagenesis (Fig. 9.7).

9.4.4.4 Genetic Lesions of DLBCL Derived from CLL and FL Transformation

The genomic analysis of sequential biopsies of CLL and FL pre- and post-transformation to DLBCL have provided insights onto the mechanisms underlying these transformation processes. These studies have revealed that the transformation of CLL into DLBCL (called Richter Syndrome) derives from the dominant CLL clone through a linear pattern, involving the maintenance of the CLL-associated lesions and the acquisition of new ones, namely *NOTCH1* mutations, *CDKN2A*/*CDKN2B* loss, *TP53* loss, and *MYC* translocations [60]. Conversely, FL and tFL derive from a common mutated precursor clone by divergent evolution involving the disruption of distinct genes and pathways; lesions specifically acquired at transformation include *CDKN2A*/*B* loss, *TP53* loss, *MYC* translocations, ASHM and *B2M* inactivation [61, 182]. Comparison with *de novo* DLBCL showed that, despite their morphologic resemblance, the genomic landscapes of RS and tFL are largely unique since they are characterized in part by distinct combinations of alterations otherwise not commonly observed in *de novo* DLBCL [60, 61]. Thus, the histologic diagnosis of DLBCL may include at least five genetically distinct diseases: GCB-DLBCL, ABC-DLBCL, PMBCL, tFL, and RS DLBCL. This distinction has implications for the development of targeted therapies.

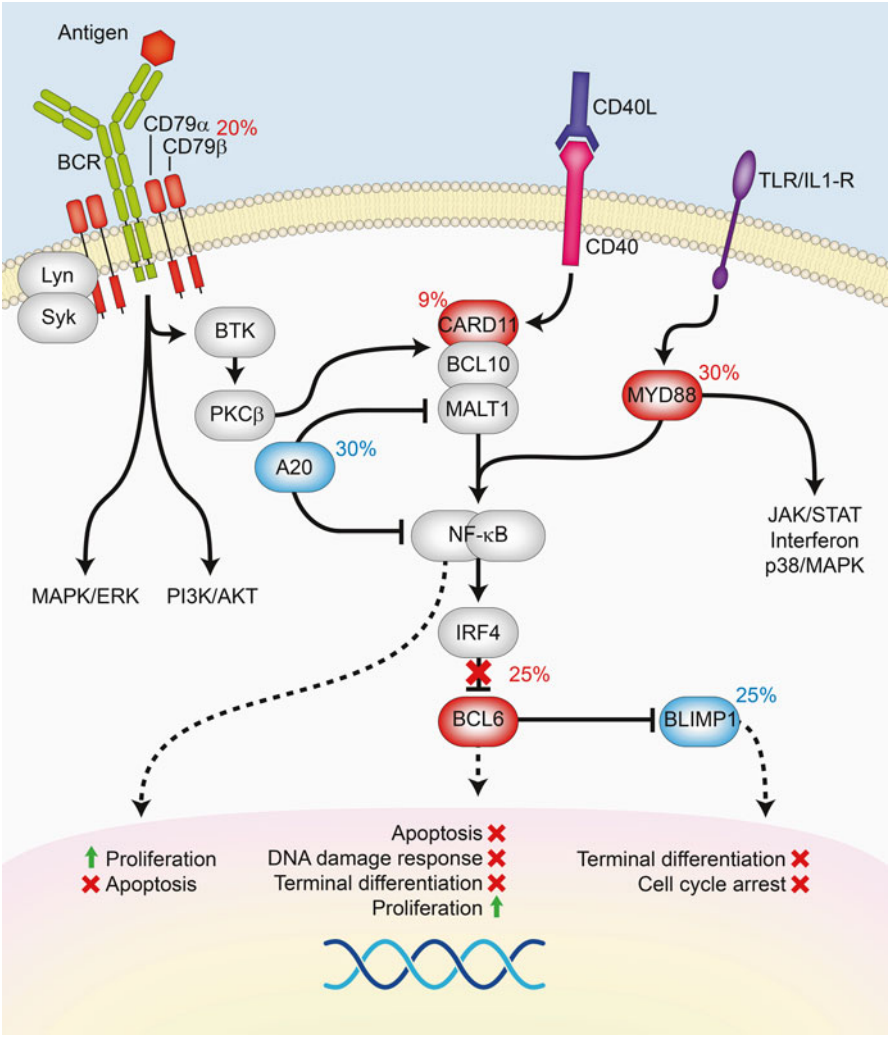

Fig. 9.7 Pathway lesions in ABC-DLBCL. Schematic representation of the signalling pathways induced following engagement of the BCR by the antigen, CD40-CD40L interaction, and activation of the TLR. These signals share the ability to activate the NF-κB pathway, leading to upregulated expression of hundreds of genes, including *IRF4* and *TNFAIP3*/*A20*. IRF4, in turn, represses BCL6, thus releasing the expression of its target PRDM1/BLIMP1. In ABC-DLBCL, multiple genetic lesions converge on this pathway, causing the constitutive activation of NF-κB, as well as chronic active BCR and JAK/STAT3 signalling, while blocking terminal B cell differentiation through mutually exclusive BCL6 deregulation and PRDM1/BLIMP1 inactivation. Genes targeted by gain-of-function mutations or translocations are in red, and genes targeted by loss-of-function genetic lesions are in blue

9.4.4.5 Genetic Lesions of PMBCL

This lymphoma displays a unique transcriptional profile that is similar to HL and suggests the derivation from post-GC thymic B cells [149–153]. One of the most common alterations in both PMBCL and HL is represented by amplification of chromosomal region 9p24, found in up to 50 % of patients [46, 183]. The amplified region encompasses multiple candidate genes, including the gene encoding for the JAK2 tyrosine kinase and the *PDL1*/*PDL2* genes, which encode for inhibitors of T-cell responses and may thus favour immune evasion of the tumour cells. Genomic breakpoints and mutations have also been described in the *CIITA* gene, encoding for the MHC class II transactivator; these lesions may reduce tumour cell immunogenicity by downregulating the expression of surface HLA class II molecules [46, 165, 184]. Analogous to HL, PMBCL patients harbour multiple genetic lesions affecting the NF-κB pathway and the JAK-STAT signalling pathway [185], including mutations of the transcription factor *STAT6*, amplifications/overexpression of *JAK2* (which promote STAT6 activation via IL3/IL4), and inactivating mutations of the STAT6 negative regulator *SOCS1*. More recently, recurrent inactivating somatic mutations of PTPN1 were reported in 22 % of PMBCL cases, where they lead to reduced phosphatase activity and increased phosphorylation of JAK-STAT pathway members [186]. Deregulation of these two signalling pathways is thus a central contributor to PMBCL pathogenesis.

References

1. Klein U, Tu Y, Stolovitzky GA, Keller JL, Haddad J Jr, Miljkovic V, Cattoretti G, Califano A, Dalla-Favera R (2003) Transcriptional analysis of the B cell germinal center reaction. Proc Natl Acad Sci U S A 100(5):2639–2644
2. MacLennan IC (1994) Germinal centers. Annu Rev Immunol 12:117–139
3. Rajewsky K (1996) Clonal selection and learning in the antibody system. Nature 381(6585):751–758
4. Allen CD, Okada T, Cyster JG (2007) Germinal-center organization and cellular dynamics. Immunity 27(2):190–202
5. Victora GD, Schwickert TA, Fooksman DR, Kamphorst AO, Meyer-Hermann M, Dustin ML, Nussenzweig MC (2010) Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. Cell 143(4):592–605
6. Neuberger MS, Ehrenstein MR, Klix N, Jolly CJ, Yelamos J, Rada C, Milstein C (1998) Monitoring and interpreting the intrinsic features of somatic hypermutation. Immunol Rev 162:107–116
7. Klein U, Dalla-Favera R (2008) Germinal centres: role in B-cell physiology and malignancy. Nat Rev Immunol 8(1):22–33
8. Liu YJ, Arpin C, de Bouteiller O, Guret C, Banchereau J, Martinez-Valdez H, Lebecque S (1996) Sequential triggering of apoptosis, somatic mutation and isotype switch during germinal center development. Semin Immunol 8(3):169–177
9. Longerich S, Basu U, Alt F, Storb U (2006) AID in somatic hypermutation and class switch recombination. Curr Opin Immunol 18(2):164–174

10. Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T (2000) Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. Cell 102(5):553–563
11. Revy P, Muto T, Levy Y, Geissmann F, Plebani A, Sanal O, Catalan N, Forveille M, Dufourcq-Labelouse R, Gennery A, Tezcan I, Ersoy F, Kayserili H, Ugazio AG, Brousse N, Muramatsu M, Notarangelo LD, Kinoshita K, Honjo T, Fischer A, Durandy A (2000) Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). Cell 102(5):565–575
12. Cattoretti G, Chang CC, Cechova K, Zhang J, Ye BH, Falini B, Louie DC, Offit K, Chaganti RS, Dalla-Favera R (1995) BCL-6 protein is expressed in germinal-center B cells. Blood 86(1):45–53
13. Chang CC, Ye BH, Chaganti RS, Dalla-Favera R (1996) BCL-6, a POZ/zinc-finger protein, is a sequence-specific transcriptional repressor. Proc Natl Acad Sci U S A 93(14):6947–6952
14. Ye BH, Cattoretti G, Shen Q, Zhang J, Hawe N, de Waard R, Leung C, Nouri-Shirazi M, Orazi A, Chaganti RS, Rothman P, Stall AM, Pandolfi PP, Dalla-Favera R (1997) The BCL-6 proto-oncogene controls germinal-centre formation and Th2- type inflammation. Nat Genet 16(2):161–170
15. Basso K, Saito M, Sumazin P, Margolin AA, Wang K, Lim WK, Kitagawa Y, Schneider C, Alvarez MJ, Califano A, Dalla-Favera R (2010) Integrated biochemical and computational approach identifies BCL6 direct target genes controlling multiple pathways in normal germinal center B cells. Blood 115(5):975–984
16. Niu H, Ye BH, Dalla-Favera R (1998) Antigen receptor signaling induces MAP kinase-mediated phosphorylation and degradation of the BCL-6 transcription factor. Genes Dev 12(13):1953–1961
17. Ci W, Polo JM, Cerchietti L, Shaknovich R, Wang L, Yang SN, Ye K, Farinha P, Horsman DE, Gascoyne RD, Elemento O, Melnick A (2009) The BCL6 transcriptional program features repression of multiple oncogenes in primary B cells and is deregulated in DLBCL. Blood 113(22):5536–5548
18. Phan RT, Dalla-Favera R (2004) The BCL6 proto-oncogene suppresses p53 expression in germinal-centre B cells. Nature 432(7017):635–639
19. Phan RT, Saito M, Basso K, Niu H, Dalla-Favera R (2005) BCL6 interacts with the transcription factor Miz-1 to suppress the cyclin-dependent kinase inhibitor p21 and cell cycle arrest in germinal center B cells. Nat Immunol 6(10):1054–1060
20. Ranuncolo SM, Polo JM, Dierov J, Singer M, Kuo T, Greally J, Green R, Carroll M, Melnick A (2007) Bcl-6 mediates the germinal center B cell phenotype and lymphomagenesis through transcriptional repression of the DNA-damage sensor ATR. Nat Immunol 8(7):705–714
21. Ranuncolo SM, Polo JM, Melnick A (2008) BCL6 represses CHEK1 and suppresses DNA damage pathways in normal and malignant B-cells. Blood Cells Mol Dis 41(1):95–99
22. Shaffer AL, Yu X, He Y, Boldrick J, Chan EP, Staudt LM (2000) BCL-6 represses genes that function in lymphocyte differentiation, inflammation, and cell cycle control. Immunity 13(2):199–212
23. Tunyaplin C, Shaffer AL, Angelin-Duclos CD, Yu X, Staudt LM, Calame KL (2004) Direct repression of prdm1 by Bcl-6 inhibits plasmacytic differentiation. J Immunol 173(2):1158–1165
24. Küppers R, Klein U, Hansmann ML, Rajewsky K (1999) Cellular origin of human B-cell lymphomas. N Engl J Med 341(20):1520–1529
25. Tsujimoto Y, Jaffe E, Cossman J, Gorham J, Nowell PC, Croce CM (1985) Clustering of breakpoints on chromosome 11 in human B-cell neoplasms with the t(11;14) chromosome translocation. Nature 315(6017):340–343
26. Küppers R, Dalla-Favera R (2001) Mechanisms of chromosomal translocation in B-cell lymphoma. Oncogene 20:5580–5594

27. Tsujimoto Y, Gorham J, Cossman J, Jaffe E, Croce CM (1985) The t(14;18) chromosome translocations involved in B-cell neoplasms result from mistakes in VDJ joining. Science 229(4720):1390–1393
28. Ramiro AR, Jankovic M, Eisenreich T, Difilippantonio S, Chen-Kiang S, Muramatsu M, Honjo T, Nussenzweig A, Nussenzweig MC (2004) AID is required for c-myc/IgH chromosome translocations in vivo. Cell 118(4):431–438
29. Robbiani DF, Bothmer A, Callen E, Reina-San-Martin B, Dorsett Y, Difilippantonio S, Bolland DJ, Chen HT, Corcoran AE, Nussenzweig A, Nussenzweig MC (2008) AID is required for the chromosomal breaks in c-myc that lead to c-myc/IgH translocations. Cell 135(6):1028–1038
30. Pasqualucci L, Bhagat G, Jankovic M, Compagno M, Smith P, Muramatsu M, Honjo T, Morse HC 3rd, Nussenzweig MC, Dalla-Favera R (2008) AID is required for germinal center-derived lymphomagenesis. Nat Genet 40(1):108–112
31. Takizawa M, Tolarova H, Li Z, Dubois W, Lim S, Callen E, Franco S, Mosaico M, Feigenbaum L, Alt FW, Nussenzweig A, Potter M, Casellas R (2008) AID expression levels determine the extent of cMyc oncogenic translocations and the incidence of B cell tumor development. J Exp Med 205(9):1949–1957
32. Akasaka H, Akasaka T, Kurata M, Ueda C, Shimizu A, Uchiyama T, Ohno H (2000) Molecular anatomy of BCL6 translocations revealed by long-distance polymerase chain reaction-based assays. Cancer Res 60(9):2335–2341
33. Chen W, Iida S, Louie DC, Dalla-Favera R, Chaganti RS (1998) Heterologous promoters fused to BCL6 by chromosomal translocations affecting band 3q27 cause its deregulated expression during B-cell differentiation. Blood 91(2):603–607
34. Ye BH, Lista F, Lo Coco F, Knowles DM, Offit K, Chaganti RS, Dalla-Favera R (1993) Alterations of a zinc finger-encoding gene, BCL-6, in diffuse large- cell lymphoma. Science 262(5134):747–750
35. Ye BH, Rao PH, Chaganti RS, Dalla-Favera R (1993) Cloning of bcl-6, the locus involved in chromosome translocations affecting band 3q27 in B-cell lymphoma. Cancer Res 53(12):2732–2735
36. Yoshida S, Kaneita Y, Aoki Y, Seto M, Mori S, Moriyama M (1999) Identification of heterologous translocation partner genes fused to the BCL6 gene in diffuse large B-cell lymphomas: 5′-RACE and LA – PCR analyses of biopsy samples. Oncogene 18(56):7994–7999
37. Baron BW, Nucifora G, McCabe N, Espinosa R 3rd, Le Beau MM, McKeithan TW (1993) Identification of the gene associated with the recurring chromosomal translocations t(3;14)(q27;q32) and t(3;22)(q27;q11) in B-cell lymphomas. Proc Natl Acad Sci U S A 90(11):5262–5266
38. Kerckaert JP, Deweindt C, Tilly H, Quief S, Lecocq G, Bastard C (1993) LAZ3, a novel zinc-finger encoding gene, is disrupted by recurring chromosome 3q27 translocations in human lymphomas. Nat Genet 5(1):66–70
39. Miki T, Kawamata N, Arai A, Ohashi K, Nakamura Y, Kato A, Hirosawa S, Aoki N (1994) Molecular cloning of the breakpoint for 3q27 translocation in B-cell lymphomas and leukemias. Blood 83(1):217–222
40. Akasaka T, Miura I, Takahashi N, Akasaka H, Yonetani N, Ohno H, Fukuhara S, Okuma M (1997) A recurring translocation, t(3;6)(q27;p21), in non-Hodgkin's lymphoma results in replacement of the 5′ regulatory region of BCL6 with a novel H4 histone gene. Cancer Res 57(1):7–12
41. Neri A, Knowles DM, Greco A, McCormick F, Dalla-Favera R (1988) Analysis of RAS oncogene mutations in human lymphoid malignancies. Proc Natl Acad Sci U S A 85(23):9268–9272
42. Houldsworth J, Mathew S, Rao PH, Dyomina K, Louie DC, Parsa N, Offit K, Chaganti RS (1996) REL proto-oncogene is frequently amplified in extranodal diffuse large cell lymphoma. Blood 87(1):25–29

43. Houldsworth J, Olshen AB, Cattoretti G, Donnelly GB, Teruya-Feldstein J, Qin J, Palanisamy N, Shen Y, Dyomina K, Petlakh M, Pan Q, Zelenetz AD, Dalla-Favera R, Chaganti RS (2004) Relationship between REL amplification, REL function, and clinical and biologic features in diffuse large B-cell lymphomas. Blood 103(5):1862–1868
44. Rao PH, Houldsworth J, Dyomina K, Parsa NZ, Cigudosa JC, Louie DC, Popplewell L, Offit K, Jhanwar SC, Chaganti RS (1998) Chromosomal and gene amplification in diffuse large B-cell lymphoma. Blood 92(1):234–240
45. Rosenwald A, Wright G, Chan WC, Connors JM, Campo E, Fisher RI, Gascoyne RD, Muller-Hermelink HK, Smeland EB, Giltnane JM, Hurt EM, Zhao H, Averett L, Yang L, Wilson WH, Jaffe ES, Simon R, Klausner RD, Powell J, Duffey PL, Longo DL, Greiner TC, Weisenburger DD, Sanger WG, Dave BJ, Lynch JC, Vose J, Armitage JO, Montserrat E, Lopez-Guillermo A, Grogan TM, Miller TP, LeBlanc M, Ott G, Kvaloy S, Delabie J, Holte H, Krajci P, Stokke T, Staudt LM (2002) The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. N Engl J Med 346(25):1937–1947
46. Green MR, Monti S, Rodig SJ, Juszczynski P, Currie T, O'Donnell E, Chapuy B, Takeyama K, Neuberg D, Golub TR, Kutok JL, Shipp MA (2010) Integrative analysis reveals selective 9p24.1 amplification, increased PD-1 ligand expression, and further induction via JAK2 in nodular sclerosing Hodgkin lymphoma and primary mediastinal large B-cell lymphoma. Blood 116:3268–3277
47. Mandelbaum J, Bhagat G, Tang H, Mo T, Brahmachary M, Shen Q, Chadburn A, Rajewsky K, Tarakhovsky A, Pasqualucci L, Dalla-Favera R (2010) BLIMP1 is a tumor suppressor gene frequently disrupted in activated B cell-like diffuse large B cell lymphoma. Cancer Cell 18(6):568–579
48. Pasqualucci L, Compagno M, Houldsworth J, Monti S, Grunn A, Nandula SV, Aster JC, Murty VV, Shipp MA, Dalla-Favera R (2006) Inactivation of the PRDM1/BLIMP1 gene in diffuse large B cell lymphoma. J Exp Med 203(2):311–317
49. Tam W, Gomez M, Chadburn A, Lee JW, Chan WC, Knowles DM (2006) Mutational analysis of PRDM1 indicates a tumor-suppressor role in diffuse large B-cell lymphomas. Blood 107(10):4090–4100
50. Compagno M, Lim WK, Grunn A, Nandula SV, Brahmachary M, Shen Q, Bertoni F, Ponzoni M, Scandurra M, Califano A, Bhagat G, Chadburn A, Dalla-Favera R, Pasqualucci L (2009) Mutations of multiple genes cause deregulation of NF-kappaB in diffuse large B-cell lymphoma. Nature 459(7247):717–721
51. Kato M, Sanada M, Kato I, Sato Y, Takita J, Takeuchi K, Niwa A, Chen Y, Nakazaki K, Nomoto J, Asakura Y, Muto S, Tamura A, Iio M, Akatsuka Y, Hayashi Y, Mori H, Igarashi T, Kurokawa M, Chiba S, Mori S, Ishikawa Y, Okamoto K, Tobinai K, Nakagama H, Nakahata T, Yoshino T, Kobayashi Y, Ogawa S (2009) Frequent inactivation of A20 in B-cell lymphomas. Nature 459(7247):712–716
52. Novak U, Rinaldi A, Kwee I, Nandula SV, Rancoita PM, Compagno M, Cerri M, Rossi D, Murty VV, Zucca E, Gaidano G, Dalla-Favera R, Pasqualucci L, Bhagat G, Bertoni F (2009) The NF-{kappa}B negative regulator TNFAIP3 (A20) is inactivated by somatic mutations and genomic deletions in marginal zone lymphomas. Blood 113(20):4918–4921
53. Schmitz R, Hansmann ML, Bohle V, Martin-Subero JI, Hartmann S, Mechtersheimer G, Klapper W, Vater I, Giefing M, Gesk S, Stanelle J, Siebert R, Kuppers R (2009) TNFAIP3 (A20) is a tumor suppressor gene in Hodgkin lymphoma and primary mediastinal B cell lymphoma. J Exp Med 206(5):981–989
54. Pasqualucci L, Dominguez-Sola D, Chiarenza A, Fabbri G, Grunn A, Trifonov V, Kasper LH, Lerach S, Tang H, Ma J, Rossi D, Chadburn A, Murty VV, Mullighan CG, Gaidano G, Rabadan R, Brindle PK, Dalla-Favera R (2011) Inactivating mutations of acetyltransferase genes in B-cell lymphoma. Nature 471(7337):189–195
55. Morin RD, Mendez-Lago M, Mungall AJ, Goya R, Mungall KL, Corbett RD, Johnson NA, Severson TM, Chiu R, Field M, Jackman S, Krzywinski M, Scott DW, Trinh DL, Tamura-Wells J, Li S, Firme MR, Rogic S, Griffith M, Chan S, Yakovenko O, Meyer IM,

Zhao EY, Smailus D, Moksa M, Chittaranjan S, Rimsza L, Brooks-Wilson A, Spinelli JJ, Ben-Neriah S, Meissner B, Woolcock B, Boyle M, McDonald H, Tam A, Zhao Y, Delaney A, Zeng T, Tse K, Butterfield Y, Birol I, Holt R, Schein J, Horsman DE, Moore R, Jones SJ, Connors JM, Hirst M, Gascoyne RD, Marra MA (2011) Frequent mutation of histone-modifying genes in non-Hodgkin lymphoma. Nature 476(7360):298–303

56. Pasqualucci L, Trifonov V, Fabbri G, Ma J, Rossi D, Chiarenza A, Wells VA, Grunn A, Messina M, Elliot O, Chan J, Bhagat G, Chadburn A, Gaidano G, Mullighan CG, Rabadan R, Dalla-Favera R (2011) Analysis of the coding genome of diffuse large B-cell lymphoma. Nat Genet 43(9):830–837
57. Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, Aldler H, Rattan S, Keating M, Rai K, Rassenti L, Kipps T, Negrini M, Bullrich F, Croce CM (2002) Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A 99(24):15524–15529
58. Dohner H, Stilgenbauer S, Benner A, Leupolt E, Krober A, Bullinger L, Dohner K, Bentz M, Lichter P (2000) Genomic aberrations and survival in chronic lymphocytic leukemia. N Engl J Med 343(26):1910–1916
59. Migliazza A, Bosch F, Komatsu H, Cayanis E, Martinotti S, Toniato E, Guccione E, Qu X, Chien M, Murty VV, Gaidano G, Inghirami G, Zhang P, Fischer S, Kalachikov SM, Russo J, Edelman I, Efstratiadis A, Dalla-Favera R (2001) Nucleotide sequence, transcription map, and mutation analysis of the 13q14 chromosomal region deleted in B-cell chronic lymphocytic leukemia. Blood 97(7):2098–2104
60. Fabbri G, Khiabanian H, Holmes AB, Wang J, Messina M, Mullighan CG, Pasqualucci L, Rabadan R, Dalla-Favera R (2013) Genetic lesions associated with chronic lymphocytic leukemia transformation to Richter syndrome. J Exp Med 210(11):2273–2288
61. Pasqualucci L, Khiabanian H, Fangazio M, Vasishtha M, Messina M, Holmes AB, Ouillette P, Trifonov V, Rossi D, Tabbo F, Ponzoni M, Chadburn A, Murty VV, Bhagat G, Gaidano G, Inghirami G, Malek SN, Rabadan R, Dalla-Favera R (2014) Genetics of follicular lymphoma transformation. Cell Rep 6(1):130–140
62. Lenz G, Wright GW, Emre NC, Kohlhammer H, Dave SS, Davis RE, Carty S, Lam LT, Shaffer AL, Xiao W, Powell J, Rosenwald A, Ott G, Muller-Hermelink HK, Gascoyne RD, Connors JM, Campo E, Jaffe ES, Delabie J, Smeland EB, Rimsza LM, Fisher RI, Weisenburger DD, Chan WC, Staudt LM (2008) Molecular subtypes of diffuse large B-cell lymphoma arise by distinct genetic pathways. Proc Natl Acad Sci U S A 105(36):13520–13525
63. Jares P, Colomer D, Campo E (2007) Genetic and molecular pathogenesis of mantle cell lymphoma: perspectives for new targeted therapeutics. Nat Rev Cancer 7(10):750–762
64. Muller PA, Vousden KH (2013) p53 mutations in cancer. Nat Cell Biol 15(1):2–8
65. Gaidano G, Ballerini P, Gong JZ, Inghirami G, Neri A, Newcomb EW, Magrath IT, Knowles DM, Dalla-Favera R (1991) p53 mutations in human lymphoid malignancies: association with Burkitt lymphoma and chronic lymphocytic leukemia. Proc Natl Acad Sci U S A 88(12):5413–5417
66. Lo Coco F, Gaidano G, Louie DC, Offit K, Chaganti RS, Dalla-Favera R (1993) p53 mutations are associated with histologic transformation of follicular lymphoma. Blood 82(8):2289–2295
67. Neuberger MS (2008) Antibody diversification by somatic mutation: from Burnet onwards. Immunol Cell Biol 86(2):124–132
68. Gordon MS, Kanegai CM, Doerr JR, Wall R (2003) Somatic hypermutation of the B cell receptor genes B29 (Igbeta, CD79b) and mb1 (Igalpha, CD79a). Proc Natl Acad Sci U S A 100(7):4126–4131
69. Pasqualucci L, Migliazza A, Fracchiolla N, William C, Neri A, Baldini L, Chaganti RS, Klein U, Kuppers R, Rajewsky K, Dalla-Favera R (1998) BCL-6 mutations in normal germinal center B cells: evidence of somatic hypermutation acting outside Ig loci. Proc Natl Acad Sci U S A 95(20):11816–11821

70. Shen HM, Peters A, Baron B, Zhu X, Storb U (1998) Mutation of BCL-6 gene in normal B cells by the process of somatic hypermutation of Ig genes. Science 280(5370):1750–1752
71. Pasqualucci L, Neumeister P, Goossens T, Nanjangud G, Chaganti RS, Kuppers R, Dalla-Favera R (2001) Hypermutation of multiple proto-oncogenes in B-cell diffuse large-cell lymphomas. Nature 412(6844):341–346
72. Cerri M, Capello D, Muti G, Rambaldi A, Paulli M, Gloghini A, Berra E, Deambrogi C, Rossi D, Franceschetti S, Conconi A, Morra E, Pasqualucci L, Carbone A, Gaidano G (2004) Aberrant somatic hypermutation in post-transplant lymphoproliferative disorders. Br J Haematol 127(3):362–364
73. Deutsch AJ, Aigelsreiter A, Staber PB, Beham A, Linkesch W, Guelly C, Brezinschek RI, Fruhwirth M, Emberger W, Buettner M, Beham-Schmid C, Neumeister P (2007) MALT lymphoma and extranodal diffuse large B-cell lymphoma are targeted by aberrant somatic hypermutation. Blood 109(8):3500–3504
74. Gaidano G, Pasqualucci L, Capello D, Berra E, Deambrogi C, Rossi D, Larocca LM, Gloghini A, Carbone A, Dalla-Favera R (2003) Aberrant somatic hypermutation in multiple subtypes of AIDS-associated non-Hodgkin lymphoma. Blood 102:1833–1841
75. Montesinos-Rongen M, Van Roost D, Schaller C, Wiestler OD, Deckert M (2004) Primary diffuse large B-cell lymphomas of the central nervous system are targeted by aberrant somatic hypermutation. Blood 103(5):1869–1875
76. Vakiani E, Basso K, Klein U, Mansukhani MM, Narayan G, Smith PM, Murty VV, Dalla-Favera R, Pasqualucci L, Bhagat G (2008) Genetic and phenotypic analysis of B-cell post-transplant lymphoproliferative disorders provides insights into disease biology. Hematol Oncol 26(4):199–211
77. Storb U, Peters A, Klotz E, Kim N, Shen HM, Hackett J, Rogerson B, Martin TE (1998) Cis-acting sequences that affect somatic hypermutation of Ig genes. Immunol Rev 162:153–160
78. Tsujimoto Y, Yunis J, Onorato-Showe L, Erikson J, Nowell PC, Croce CM (1984) Molecular cloning of the chromosomal breakpoint of B-cell lymphomas and leukemias with the t(11;14) chromosome translocation. Science 224(4656):1403–1406
79. Erikson J, Finan J, Tsujimoto Y, Nowell PC, Croce CM (1984) The chromosome 14 breakpoint in neoplastic B cells with the t(11;14) translocation involves the immunoglobulin heavy chain locus. Proc Natl Acad Sci U S A 81(13):4144–4148
80. Motokura T, Bloom T, Kim HG, Juppner H, Ruderman JV, Kronenberg HM, Arnold A (1991) A novel cyclin encoded by a bcl1-linked candidate oncogene. Nature 350(6318):512–515
81. Rosenberg CL, Wong E, Petty EM, Bale AE, Tsujimoto Y, Harris NL, Arnold A (1991) PRAD1, a candidate BCL1 oncogene: mapping and expression in centrocytic lymphoma. Proc Natl Acad Sci U S A 88(21):9638–9642
82. Withers DA, Harvey RC, Faust JB, Melnyk O, Carey K, Meeker TC (1991) Characterization of a candidate bcl-1 gene. Mol Cell Biol 11(10):4846–4853
83. Seto M, Yamamoto K, Iida S, Akao Y, Utsumi KR, Kubonishi I, Miyoshi I, Ohtsuki T, Yawata Y, Namba M et al (1992) Gene rearrangement and overexpression of PRAD1 in lymphoid malignancy with t(11;14)(q13;q32) translocation. Oncogene 7(7):1401–1406
84. Komatsu H, Iida S, Yamamoto K, Mikuni C, Nitta M, Takahashi T, Ueda R, Seto M (1994) A variant chromosome translocation at 11q13 identifying PRAD1/cyclin D1 as the BCL-1 gene. Blood 84(4):1226–1231
85. Wiestner A, Tehrani M, Chiorazzi M, Wright G, Gibellini F, Nakayama K, Liu H, Rosenwald A, Muller-Hermelink HK, Ott G, Chan WC, Greiner TC, Weisenburger DD, Vose J, Armitage JO, Gascoyne RD, Connors JM, Campo E, Montserrat E, Bosch F, Smeland EB, Kvaloy S, Holte H, Delabie J, Fisher RI, Grogan TM, Miller TP, Wilson WH, Jaffe ES, Staudt LM (2007) Point mutations and genomic deletions in CCND1 create stable truncated cyclin D1 mRNAs that are associated with increased proliferation rate and shorter survival. Blood 109(11):4599–4606

86. Bodrug SE, Warner BJ, Bath ML, Lindeman GJ, Harris AW, Adams JM (1994) Cyclin D1 transgene impedes lymphocyte maturation and collaborates in lymphomagenesis with the myc gene. Embo J 13(9):2124–2130
87. Lovec H, Grzeschiczek A, Kowalski MB, Moroy T (1994) Cyclin D1/bcl-1 cooperates with myc genes in the generation of B-cell lymphoma in transgenic mice. Embo J 13(15):3487–3495
88. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J, Vardiman JW (2008) WHO classification of tumours of haematopoietic and lymphoid tissues. World Health Organization of Tumours. International Agency for Research on Cancer (IARC), Lyon
89. Schaffner C, Idler I, Stilgenbauer S, Dohner H, Lichter P (2000) Mantle cell lymphoma is characterized by inactivation of the ATM gene. Proc Natl Acad Sci U S A 97(6):2773–2778
90. Louie DC, Offit K, Jaslow R, Parsa NZ, Murty VV, Schluger A, Chaganti RS (1995) p53 overexpression as a marker of poor prognosis in mantle cell lymphomas with t(11;14)(q13;q32). Blood 86(8):2892–2899
91. Pinyol M, Hernandez L, Cazorla M, Balbin M, Jares P, Fernandez PL, Montserrat E, Cardesa A, Lopez-Otin C, Campo E (1997) Deletions and loss of expression of p16INK4a and p21Waf1 genes are associated with aggressive variants of mantle cell lymphomas. Blood 89(1):272–280
92. Bea S, Valdes-Mas R, Navarro A, Salaverria I, Martin-Garcia D, Jares P, Gine E, Pinyol M, Royo C, Nadeu F, Conde L, Juan M, Clot G, Vizan P, Di Croce L, Puente DA, Lopez-Guerra M, Moros A, Roue G, Aymerich M, Villamor N, Colomo L, Martinez A, Valera A, Martin-Subero JI, Amador V, Hernandez L, Rozman M, Enjuanes A, Forcada P, Muntanola A, Hartmann EM, Calasanz MJ, Rosenwald A, Ott G, Hernandez-Rivas JM, Klapper W, Siebert R, Wiestner A, Wilson WH, Colomer D, Lopez-Guillermo A, Lopez-Otin C, Puente XS, Campo E (2013) Landscape of somatic mutations and clonal evolution in mantle cell lymphoma. Proc Natl Acad Sci U S A 110(45):18250–18255
93. Kridel R, Meissner B, Rogic S, Boyle M, Telenius A, Woolcock B, Gunawardana J, Jenkins C, Cochrane C, Ben-Neriah S, Tan K, Morin RD, Opat S, Sehn LH, Connors JM, Marra MA, Weng AP, Steidl C, Gascoyne RD (2012) Whole transcriptome sequencing reveals recurrent NOTCH1 mutations in mantle cell lymphoma. Blood 119(9):1963–1971
94. Bea S, Tort F, Pinyol M, Puig X, Hernandez L, Hernandez S, Fernandez PL, van Lohuizen M, Colomer D, Campo E (2001) BMI-1 gene amplification and overexpression in hematological malignancies occur mainly in mantle cell lymphomas. Cancer Res 61(6):2409–2412
95. Chapman CJ, Mockridge CI, Rowe M, Rickinson AB, Stevenson FK (1995) Analysis of VH genes used by neoplastic B cells in endemic Burkitt's lymphoma shows somatic hypermutation and intraclonal heterogeneity. Blood 85(8):2176–2181
96. Chapman CJ, Zhou JX, Gregory C, Rickinson AB, Stevenson FK (1996) VH and VL gene analysis in sporadic Burkitt's lymphoma shows somatic hypermutation, intraclonal heterogeneity, and a role for antigen selection. Blood 88(9):3562–3568
97. Tamaru J, Hummel M, Marafioti T, Kalvelage B, Leoncini L, Minacci C, Tosi P, Wright D, Stein H (1995) Burkitt's lymphomas express VH genes with a moderate number of antigen-selected somatic mutations. Am J Pathol 147(5):1398–1407
98. Dave SS, Fu K, Wright GW, Lam LT, Kluin P, Boerma EJ, Greiner TC, Weisenburger DD, Rosenwald A, Ott G, Muller-Hermelink HK, Gascoyne RD, Delabie J, Rimsza LM, Braziel RM, Grogan TM, Campo E, Jaffe ES, Dave BJ, Sanger W, Bast M, Vose JM, Armitage JO, Connors JM, Smeland EB, Kvaloy S, Holte H, Fisher RI, Miller TP, Montserrat E, Wilson WH, Bahl M, Zhao H, Yang L, Powell J, Simon R, Chan WC, Staudt LM (2006) Molecular diagnosis of Burkitt's lymphoma. N Engl J Med 354(23):2431–2442
99. Hummel M, Bentink S, Berger H, Klapper W, Wessendorf S, Barth TF, Bernd HW, Cogliatti SB, Dierlamm J, Feller AC, Hansmann ML, Haralambieva E, Harder L, Hasenclever D, Kuhn M, Lenze D, Lichter P, Martin-Subero JI, Moller P, Muller-Hermelink HK, Ott G, Parwaresch RM, Pott C, Rosenwald A, Rosolowski M, Schwaenen C, Sturzenhofecker B, Szczepanowski M, Trautmann H, Wacker HH, Spang R, Loeffler M, Trumper L, Stein H, Siebert R (2006) A biologic definition of Burkitt's lymphoma from transcriptional and genomic profiling. N Engl J Med 354(23):2419–2430

100. Dalla-Favera R, Bregni M, Erikson J, Patterson D, Gallo RC, Croce CM (1982) Human c-myc onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. Proc Natl Acad Sci U S A 79(24):7824–7827
101. Dalla-Favera R, Martinotti S, Gallo RC, Erikson J, Croce CM (1983) Translocation and rearrangements of the c-myc oncogene locus in human undifferentiated B-cell lymphomas. Science 219(4587):963–967
102. Dalla-Favera R (1993) Chromosomal translocations involving the c-myc oncogene in lymphoid neoplasia. In: Kirsch IR (ed) The causes and consequences of chromosomal aberrations. CRC Press, Boca Raton, p 312
103. Taub R, Kirsch I, Morton C, Lenoir G, Swan D, Tronick S, Aaronson S, Leder P (1982) Translocation of the c-myc gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells. Proc Natl Acad Sci U S A 79(24):7837–7841
104. Davis M, Malcolm S, Rabbitts TH (1984) Chromosome translocation can occur on either side of the c-myc oncogene in Burkitt lymphoma cells. Nature 308(5956):286–288
105. Neri A, Barriga F, Knowles DM, Magrath IT, Dalla-Favera R (1988) Different regions of the immunoglobulin heavy-chain locus are involved in chromosomal translocations in distinct pathogenetic forms of Burkitt lymphoma. Proc Natl Acad Sci U S A 85(8):2748–2752
106. Pelicci PG, Knowles DM 2nd, Magrath I, Dalla-Favera R (1986) Chromosomal breakpoints and structural alterations of the c-myc locus differ in endemic and sporadic forms of Burkitt lymphoma. Proc Natl Acad Sci U S A 83(9):2984–2988
107. ar-Rushdi A, Nishikura K, Erikson J, Watt R, Rovera G, Croce CM (1983) Differential expression of the translocated and the untranslocated c-myc oncogene in Burkitt lymphoma. Science 222(4622):390–393
108. Hayday AC, Gillies SD, Saito H, Wood C, Wiman K, Hayward WS, Tonegawa S (1984) Activation of a translocated human c-myc gene by an enhancer in the immunoglobulin heavy-chain locus. Nature 307(5949):334–340
109. Rabbitts TH, Forster A, Baer R, Hamlyn PH (1983) Transcription enhancer identified near the human C mu immunoglobulin heavy chain gene is unavailable to the translocated c-myc gene in a Burkitt lymphoma. Nature 306(5945):806–809
110. Dominguez-Sola D, Victora GD, Ying CY, Phan RT, Saito M, Nussenzweig MC, Dalla-Favera R (2012) The proto-oncogene MYC is required for selection in the germinal center and cyclic reentry. Nat Immunol 13(11):1083–1091
111. Cesarman E, Dalla-Favera R, Bentley D, Groudine M (1987) Mutations in the first exon are associated with altered transcription of c-myc in Burkitt lymphoma. Science 238(4831):1272–1275
112. Bhatia K, Huppi K, Spangler G, Siwarski D, Iyer R, Magrath I (1993) Point mutations in the c-Myc transactivation domain are common in Burkitt's lymphoma and mouse plasmacytomas. Nat Genet 5(1):56–61
113. Bhatia K, Spangler G, Gaidano G, Hamdy N, Dalla-Favera R, Magrath I (1994) Mutations in the coding region of c-myc occur frequently in acquired immunodeficiency syndrome-associated lymphomas. Blood 84(3):883–888
114. Gu W, Bhatia K, Magrath IT, Dang CV, Dalla-Favera R (1994) Binding and suppression of the Myc transcriptional activation domain by p107. Science 264(5156):251–254
115. Gregory MA, Hann SR (2000) c-Myc proteolysis by the ubiquitin-proteasome pathway: stabilization of c-Myc in Burkitt's lymphoma cells. Mol Cell Biol 20(7):2423–2435
116. Hemann MT, Bric A, Teruya-Feldstein J, Herbst A, Nilsson JA, Cordon-Cardo C, Cleveland JL, Tansey WP, Lowe SW (2005) Evasion of the p53 tumour surveillance network by tumour-derived MYC mutants. Nature 436(7052):807–811
117. Grandori C, Cowley SM, James LP, Eisenman RN (2000) The Myc/Max/Mad network and the transcriptional control of cell behavior. Annu Rev Cell Dev Biol 16:653–699
118. Meyer N, Penn LZ (2008) Reflecting on 25 years with MYC. Nat Rev Cancer 8(12):976–990
119. Dang CV, O'Donnell KA, Zeller KI, Nguyen T, Osthus RC, Li F (2006) The c-Myc target gene network. Semin Cancer Biol 16(4):253–264

120. Felsher DW, Bishop JM (1999) Transient excess of MYC activity can elicit genomic instability and tumorigenesis. Proc Natl Acad Sci U S A 96(7):3940–3944
121. Adams JM, Harris AW, Pinkert CA, Corcoran LM, Alexander WS, Cory S, Palmiter RD, Brinster RL (1985) The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. Nature 318(6046):533–538
122. Kovalchuk AL, Qi CF, Torrey TA, Taddesse-Heath L, Feigenbaum L, Park SS, Gerbitz A, Klobeck G, Hoertnagel K, Polack A, Bornkamm GW, Janz S, Morse HC 3rd (2000) Burkitt lymphoma in the mouse. J Exp Med 192(8):1183–1190
123. Sander S, Calado DP, Srinivasan L, Kochert K, Zhang B, Rosolowski M, Rodig SJ, Holzmann K, Stilgenbauer S, Siebert R, Bullinger L, Rajewsky K (2012) Synergy between PI3K signaling and MYC in Burkitt lymphomagenesis. Cancer Cell 22(2):167–179
124. Schmitz R, Young RM, Ceribelli M, Jhavar S, Xiao W, Zhang M, Wright G, Shaffer AL, Hodson DJ, Buras E, Liu X, Powell J, Yang Y, Xu W, Zhao H, Kohlhammer H, Rosenwald A, Kluin P, Muller-Hermelink HK, Ott G, Gascoyne RD, Connors JM, Rimsza LM, Campo E, Jaffe ES, Delabie J, Smeland EB, Ogwang MD, Reynolds SJ, Fisher RI, Braziel RM, Tubbs RR, Cook JR, Weisenburger DD, Chan WC, Pittaluga S, Wilson W, Waldmann TA, Rowe M, Mbulaiteye SM, Rickinson AB, Staudt LM (2012) Burkitt lymphoma pathogenesis and therapeutic targets from structural and functional genomics. Nature 490(7418):116–120
125. Martinez-Delgado B, Robledo M, Arranz E, Osorio A, Garcia MJ, Echezarreta G, Rivas C, Benitez J (1998) Hypermethylation of p15/ink4b/MTS2 gene is differentially implicated among non-Hodgkin's lymphomas. Leukemia 12(6):937–941
126. Gaidano G, Hauptschein RS, Parsa NZ, Offit K, Rao PH, Lenoir G, Knowles DM, Chaganti RS, Dalla-Favera R (1992) Deletions involving two distinct regions of 6q in B-cell non-Hodgkin lymphoma. Blood 80(7):1781–1787
127. zur Hausen H, Schulte-Holthausen H, Klein G, Henle W, Henle G, Clifford P, Santesson L (1970) EBV DNA in biopsies of Burkitt tumours and anaplastic carcinomas of the nasopharynx. Nature 228(276):1056–1058
128. Lombardi L, Newcomb EW, Dalla-Favera R (1987) Pathogenesis of Burkitt lymphoma: expression of an activated c-myc oncogene causes the tumorigenic conversion of EBV-infected human B lymphoblasts. Cell 49(2):161–170
129. Neri A, Barriga F, Inghirami G, Knowles DM, Neequaye J, Magrath IT, Dalla-Favera R (1991) Epstein-Barr virus infection precedes clonal expansion in Burkitt's and acquired immunodeficiency syndrome-associated lymphoma. Blood 77(5):1092–1095
130. Prevot S, Hamilton-Dutoit S, Audouin J, Walter P, Pallesen G, Diebold J (1992) Analysis of African Burkitt's and high-grade B cell non-Burkitt's lymphoma for Epstein-Barr virus genomes using in situ hybridization. Br J Haematol 80(1):27–32
131. Thorley-Lawson DA, Allday MJ (2008) The curious case of the tumour virus: 50 years of Burkitt's lymphoma. Nat Rev Microbiol 6(12):913–924
132. Kridel R, Sehn LH, Gascoyne RD (2012) Pathogenesis of follicular lymphoma. J Clin Invest 122(10):3424–3431
133. Montoto S, Fitzgibbon J (2011) Transformation of indolent B-cell lymphomas. J Clin Oncol 29(14):1827–1834
134. Bakhshi A, Jensen JP, Goldman P, Wright JJ, McBride OW, Epstein AL, Korsmeyer SJ (1985) Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around JH on chromosome 14 and near a transcriptional unit on 18. Cell 41(3):899–906
135. Cleary ML, Sklar J (1985) Nucleotide sequence of a t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint-cluster region near a transcriptionally active locus on chromosome 18. Proc Natl Acad Sci U S A 82(21):7439–7443
136. Cleary ML, Smith SD, Sklar J (1986) Cloning and structural analysis of cDNAs for bcl-2 and a hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation. Cell 47(1):19–28
137. Ott G, Katzenberger T, Lohr A, Kindelberger S, Rudiger T, Wilhelm M, Kalla J, Rosenwald A, Muller JG, Ott MM, Muller-Hermelink HK (2002) Cytomorphologic, immunohistochem-

ical, and cytogenetic profiles of follicular lymphoma: 2 types of follicular lymphoma grade 3. Blood 99(10):3806–3812
138. Tsujimoto Y, Finger LR, Yunis J, Nowell PC, Croce CM (1984) Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. Science 226(4678):1097–1099
139. Cleary ML, Galili N, Sklar J (1986) Detection of a second t(14;18) breakpoint cluster region in human follicular lymphomas. J Exp Med 164(1):315–320
140. Graninger WB, Seto M, Boutain B, Goldman P, Korsmeyer SJ (1987) Expression of Bcl-2 and Bcl-2-Ig fusion transcripts in normal and neoplastic cells. J Clin Invest 80(5):1512–1515
141. Ngan BY, Chen-Levy Z, Weiss LM, Warnke RA, Cleary ML (1988) Expression in non-Hodgkin's lymphoma of the bcl-2 protein associated with the t(14;18) chromosomal translocation. N Engl J Med 318(25):1638–1644
142. Petrovic AS, Young RL, Hilgarth B, Ambros P, Korsmeyer SJ, Jaeger U (1998) The Ig heavy chain 3′ end confers a posttranscriptional processing advantage to Bcl-2-IgH fusion RNA in t(14;18) lymphoma. Blood 91(10):3952–3961
143. Saito M, Novak U, Piovan E, Basso K, Sumazin P, Schneider C, Crespo M, Shen Q, Bhagat G, Califano A, Chadburn A, Pasqualucci L, Dalla-Favera R (2009) BCL6 suppression of BCL2 via Miz1 and its disruption in diffuse large B cell lymphoma. Proc Natl Acad Sci U S A 106(27):11294–11299
144. Buchonnet G, Jardin F, Jean N, Bertrand P, Parmentier F, Tison S, Lepretre S, Contentin N, Lenain P, Stamatoullas-Bastard A, Tilly H, Bastard C (2002) Distribution of BCL2 breakpoints in follicular lymphoma and correlation with clinical features: specific subtypes or same disease? Leukemia 16(9):1852–1856
145. Yano T, Jaffe ES, Longo DL, Raffeld M (1992) MYC rearrangements in histologically progressed follicular lymphomas. Blood 80(3):758–767
146. Ichikawa A, Hotta T, Takagi N, Tsushita K, Kinoshita T, Nagai H, Murakami Y, Hayashi K, Saito H (1992) Mutations of p53 gene and their relation to disease progression in B-cell lymphoma. Blood 79(10):2701–2707
147. O'Shea D, O'Riain C, Taylor C, Waters R, Carlotti E, Macdougall F, Gribben J, Rosenwald A, Ott G, Rimsza LM, Smeland EB, Johnson N, Campo E, Greiner TC, Chan WC, Gascoyne RD, Wright G, Staudt LM, Lister TA, Fitzgibbon J (2008) The presence of TP53 mutation at diagnosis of follicular lymphoma identifies a high-risk group of patients with shortened time to disease progression and poorer overall survival. Blood 112(8):3126–3129
148. Sander CA, Yano T, Clark HM, Harris C, Longo DL, Jaffe ES, Raffeld M (1993) p53 mutation is associated with progression in follicular lymphomas. Blood 82(7):1994–2004
149. Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, Boldrick JC, Sabet H, Tran T, Yu X, Powell JI, Yang L, Marti GE, Moore T, Hudson J, Lu L, Lewis DB, Tibshirani R, Sherlock G, Chan WC, Greiner TC, Weisenburger DD, Armitage JO, Warnke R, Staudt LM et al (2000) Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature 403(6769):503–511
150. Wright G, Tan B, Rosenwald A, Hurt EH, Wiestner A, Staudt LM (2003) A gene expression-based method to diagnose clinically distinct subgroups of diffuse large B cell lymphoma. Proc Natl Acad Sci U S A 100(17):9991–9996
151. Savage KJ, Monti S, Kutok JL, Cattoretti G, Neuberg D, De Leval L, Kurtin P, Dal Cin P, Ladd C, Feuerhake F, Aguiar RC, Li S, Salles G, Berger F, Jing W, Pinkus GS, Habermann T, Dalla-Favera R, Harris NL, Aster JC, Golub TR, Shipp MA (2003) The molecular signature of mediastinal large B-cell lymphoma differs from that of other diffuse large B-cell lymphomas and shares features with classical Hodgkin lymphoma. Blood 102(12):3871–3879
152. Rosenwald A, Wright G, Leroy K, Yu X, Gaulard P, Gascoyne RD, Chan WC, Zhao T, Haioun C, Greiner TC, Weisenburger DD, Lynch JC, Vose J, Armitage JO, Smeland EB, Kvaloy S, Holte H, Delabie J, Campo E, Montserrat E, Lopez-Guillermo A, Ott G, Muller-Hermelink HK, Connors JM, Braziel R, Grogan TM, Fisher RI, Miller TP, LeBlanc M, Chiorazzi M, Zhao H, Yang L, Powell J, Wilson WH, Jaffe ES, Simon R, Klausner RD, Staudt LM (2003)

Molecular diagnosis of primary mediastinal B cell lymphoma identifies a clinically favorable subgroup of diffuse large B cell lymphoma related to Hodgkin lymphoma. J Exp Med 198(6):851–862
153. Victora GD, Dominguez-Sola D, Holmes AB, Deroubaix S, Dalla-Favera R, Nussenzweig MC (2012) Identification of human germinal center light and dark zone cells and their relationship to human B-cell lymphomas. Blood 120(11):2240–2248
154. Lohr JG, Stojanov P, Lawrence MS, Auclair D, Chapuy B, Sougnez C, Cruz-Gordillo P, Knoechel B, Asmann YW, Slager SL, Novak AJ, Dogan A, Ansell SM, Link BK, Zou L, Gould J, Saksena G, Stransky N, Rangel-Escareno C, Fernandez-Lopez JC, Hidalgo-Miranda A, Melendez-Zajgla J, Hernandez-Lemus E, Schwarz-Cruz y Celis A, Imaz-Rosshandler I, Ojesina AI, Jung J, Pedamallu CS, Lander ES, Habermann TM, Cerhan JR, Shipp MA, Getz G, Golub TR (2012) Discovery and prioritization of somatic mutations in diffuse large B-cell lymphoma (DLBCL) by whole-exome sequencing. Proc Natl Acad Sci U S A 109(10):3879–3884
155. Lo Coco F, Ye BH, Lista F, Corradini P, Offit K, Knowles DM, Chaganti RS, Dalla-Favera R (1994) Rearrangements of the BCL6 gene in diffuse large cell non-Hodgkin's lymphoma. Blood 83(7):1757–1759
156. Offit K, Jhanwar S, Ebrahim SA, Filippa D, Clarkson BD, Chaganti RS (1989) t(3;22)(q27;q11): a novel translocation associated with diffuse non-Hodgkin's lymphoma. Blood 74(6):1876–1879
157. Offit K, Wong G, Filippa DA, Tao Y, Chaganti RS (1991) Cytogenetic analysis of 434 consecutively ascertained specimens of non-Hodgkin's lymphoma: clinical correlations. Blood 77(7):1508–1515
158. Iqbal J, Greiner TC, Patel K, Dave BJ, Smith L, Ji J, Wright G, Sanger WG, Pickering DL, Jain S, Horsman DE, Shen Y, Fu K, Weisenburger DD, Hans CP, Campo E, Gascoyne RD, Rosenwald A, Jaffe ES, Delabie J, Rimsza L, Ott G, Muller-Hermelink HK, Connors JM, Vose JM, McKeithan T, Staudt LM, Chan WC (2007) Distinctive patterns of BCL6 molecular alterations and their functional consequences in different subgroups of diffuse large B-cell lymphoma. Leukemia 21(11):2332–2343
159. Ye BH, Chaganti S, Chang CC, Niu H, Corradini P, Chaganti RS, Dalla-Favera R (1995) Chromosomal translocations cause deregulated BCL6 expression by promoter substitution in B cell lymphoma. Embo J 14(24):6209–6217
160. Ying CY, Dominguez-Sola D, Fabi M, Lorenz IC, Hussein S, Bansal M, Califano A, Pasqualucci L, Basso K, Dalla-Favera R (2013) MEF2B mutations lead to deregulated expression of the oncogene BCL6 in diffuse large B cell lymphoma. Nat Immunol 14(10):1084–1092
161. Bereshchenko OR, Gu W, Dalla-Favera R (2002) Acetylation inactivates the transcriptional repressor BCL6. Nat Genet 32(4):606–613
162. Duan S, Cermak L, Pagan JK, Rossi M, Martinengo C, di Celle PF, Chapuy B, Shipp M, Chiarle R, Pagano M (2012) FBXO11 targets BCL6 for degradation and is inactivated in diffuse large B-cell lymphomas. Nature 481(7379):90–93
163. Cattoretti G, Pasqualucci L, Ballon G, Tam W, Nandula SV, Shen Q, Mo T, Murty VV, Dalla-Favera R (2005) Deregulated BCL6 expression recapitulates the pathogenesis of human diffuse large B cell lymphomas in mice. Cancer Cell 7(5):445–455
164. Challa-Malladi M, Lieu YK, Califano O, Holmes AB, Bhagat G, Murty VV, Dominguez-Sola D, Pasqualucci L, Dalla-Favera R (2011) Combined genetic inactivation of beta2-microglobulin and CD58 reveals frequent escape from immune recognition in diffuse large B cell lymphoma. Cancer Cell 20(6):728–740
165. Steidl C, Shah SP, Woolcock BW, Rui L, Kawahara M, Farinha P, Johnson NA, Zhao Y, Telenius A, Neriah SB, McPherson A, Meissner B, Okoye UC, Diepstra A, van den Berg A, Sun M, Leung G, Jones SJ, Connors JM, Huntsman DG, Savage KJ, Rimsza LM, Horsman DE, Staudt LM, Steidl U, Marra MA, Gascoyne RD (2011) MHC class II transactivator CIITA is a recurrent gene fusion partner in lymphoid cancers. Nature 471(7338):377–381

166. Khodabakhshi AH, Morin RD, Fejes AP, Mungall AJ, Mungall KL, Bolger-Munro M, Johnson NA, Connors JM, Gascoyne RD, Marra MA, Birol I, Jones SJ (2012) Recurrent targets of aberrant somatic hypermutation in lymphoma. Oncotarget 3(11):1308–1319
167. Iqbal J, Sanger WG, Horsman DE, Rosenwald A, Pickering DL, Dave B, Dave S, Xiao L, Cao K, Zhu Q, Sherman S, Hans CP, Weisenburger DD, Greiner TC, Gascoyne RD, Ott G, Muller-Hermelink HK, Delabie J, Braziel RM, Jaffe ES, Campo E, Lynch JC, Connors JM, Vose JM, Armitage JO, Grogan TM, Staudt LM, Chan WC (2004) BCL2 translocation defines a unique tumor subset within the germinal center B-cell-like diffuse large B-cell lymphoma. Am J Pathol 165(1):159–166
168. Kawasaki C, Ohshim K, Suzumiya J, Kanda M, Tsuchiya T, Tamura K, Kikuchi M (2001) Rearrangements of bcl-1, bcl-2, bcl-6, and c-myc in diffuse large B-cell lymphomas. Leuk Lymphoma 42(5):1099–1106
169. Ladanyi M, Offit K, Jhanwar SC, Filippa DA, Chaganti RS (1991) MYC rearrangement and translocations involving band 8q24 in diffuse large cell lymphomas. Blood 77(5):1057–1063
170. Morin RD, Johnson NA, Severson TM, Mungall AJ, An J, Goya R, Paul JE, Boyle M, Woolcock BW, Kuchenbauer F, Yap D, Humphries RK, Griffith OL, Shah S, Zhu H, Kimbara M, Shashkin P, Charlot JF, Tcherpakov M, Corbett R, Tam A, Varhol R, Smailus D, Moksa M, Zhao Y, Delaney A, Qian H, Birol I, Schein J, Moore R, Holt R, Horsman DE, Connors JM, Jones S, Aparicio S, Hirst M, Gascoyne RD, Marra MA (2010) Somatic mutations altering EZH2 (Tyr641) in follicular and diffuse large B-cell lymphomas of germinal-center origin. Nat Genet 42(2):181–185
171. Pfeifer M, Grau M, Lenze D, Wenzel SS, Wolf A, Wollert-Wulf B, Dietze K, Nogai H, Storek B, Madle H, Dorken B, Janz M, Dirnhofer S, Lenz P, Hummel M, Tzankov A, Lenz G (2013) PTEN loss defines a PI3K/AKT pathway-dependent germinal center subtype of diffuse large B-cell lymphoma. Proc Natl Acad Sci U S A 110(30):12420–12425
172. Pasqualucci L, Migliazza A, Basso K, Houldsworth J, Chaganti RS, Dalla-Favera R (2003) Mutations of the BCL6 proto-oncogene disrupt its negative autoregulation in diffuse large B-cell lymphoma. Blood 101(8):2914–2923
173. Wang X, Li Z, Naganuma A, Ye BH (2002) Negative autoregulation of BCL-6 is bypassed by genetic alterations in diffuse large B cell lymphomas. Proc Natl Acad Sci U S A 99(23):15018–15023
174. Capello D, Vitolo U, Pasqualucci L, Quattrone S, Migliaretti G, Fassone L, Ariatti C, Vivenza D, Gloghini A, Pastore C, Lanza C, Nomdedeu J, Botto B, Freilone R, Buonaiuto D, Zagonel V, Gallo E, Palestro G, Saglio G, Dalla-Favera R, Carbone A, Gaidano G (2000) Distribution and pattern of BCL-6 mutations throughout the spectrum of B-cell neoplasia. Blood 95(2):651–659
175. Migliazza A, Martinotti S, Chen W, Fusco C, Ye BH, Knowles DM, Offit K, Chaganti RS, Dalla-Favera R (1995) Frequent somatic hypermutation of the 5′ noncoding region of the BCL6 gene in B-cell lymphoma. Proc Natl Acad Sci U S A 92(26):12520–12524
176. Shen HM, Michael N, Kim N, Storb U (2000) The TATA binding protein, c-Myc and survivin genes are not somatically hypermutated, while Ig and BCL6 genes are hypermutated in human memory B cells. Int Immunol 12(7):1085–1093
177. Saito M, Gao J, Basso K, Kitagawa Y, Smith PM, Bhagat G, Pernis A, Pasqualucci L, Dalla-Favera R (2007) A signaling pathway mediating downregulation of BCL6 in germinal center B cells is blocked by BCL6 gene alterations in B cell lymphoma. Cancer Cell 12(3):280–292
178. Lenz G, Davis RE, Ngo VN, Lam L, George TC, Wright GW, Dave SS, Zhao H, Xu W, Rosenwald A, Ott G, Muller-Hermelink HK, Gascoyne RD, Connors JM, Rimsza LM, Campo E, Jaffe ES, Delabie J, Smeland EB, Fisher RI, Chan WC, Staudt LM (2008) Oncogenic CARD11 mutations in human diffuse large B cell lymphoma. Science 319(5870):1676–1679

179. Ngo VN, Young RM, Schmitz R, Jhavar S, Xiao W, Lim KH, Kohlhammer H, Xu W, Yang Y, Zhao H, Shaffer AL, Romesser P, Wright G, Powell J, Rosenwald A, Muller-Hermelink HK, Ott G, Gascoyne RD, Connors JM, Rimsza LM, Campo E, Jaffe ES, Delabie J, Smeland EB, Fisher RI, Braziel RM, Tubbs RR, Cook JR, Weisenburger DD, Chan WC, Staudt LM (2011) Oncogenically active MYD88 mutations in human lymphoma. Nature 470:115–119
180. Davis RE, Ngo VN, Lenz G, Tolar P, Young RM, Romesser PB, Kohlhammer H, Lamy L, Zhao H, Yang Y, Xu W, Shaffer AL, Wright G, Xiao W, Powell J, Jiang JK, Thomas CJ, Rosenwald A, Ott G, Muller-Hermelink HK, Gascoyne RD, Connors JM, Johnson NA, Rimsza LM, Campo E, Jaffe ES, Wilson WH, Delabie J, Smeland EB, Fisher RI, Braziel RM, Tubbs RR, Cook JR, Weisenburger DD, Chan WC, Pierce SK, Staudt LM (2010) Chronic active B-cell-receptor signalling in diffuse large B-cell lymphoma. Nature 463(7277):88–92
181. Shapiro-Shelef M, Lin KI, McHeyzer-Williams LJ, Liao J, McHeyzer-Williams MG, Calame K (2003) Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. Immunity 19(4):607–620
182. Okosun J, Bodor C, Wang J, Araf S, Yang CY, Pan C, Boller S, Cittaro D, Bozek M, Iqbal S, Matthews J, Wrench D, Marzec J, Tawana K, Popov N, O'Riain C, O'Shea D, Carlotti E, Davies A, Lawrie CH, Matolcsy A, Calaminici M, Norton A, Byers RJ, Mein C, Stupka E, Lister TA, Lenz G, Montoto S, Gribben JG, Fan Y, Grosschedl R, Chelala C, Fitzgibbon J (2014) Integrated genomic analysis identifies recurrent mutations and evolution patterns driving the initiation and progression of follicular lymphoma. Nat Genet 46(2):176–181
183. Joos S, Kupper M, Ohl S, von Bonin F, Mechtersheimer G, Bentz M, Marynen P, Moller P, Pfreundschuh M, Trumper L, Lichter P (2000) Genomic imbalances including amplification of the tyrosine kinase gene JAK2 in CD30+ Hodgkin cells. Cancer Res 60(3):549–552
184. Rui L, Emre NC, Kruhlak MJ, Chung HJ, Steidl C, Slack G, Wright GW, Lenz G, Ngo VN, Shaffer AL, Xu W, Zhao H, Yang Y, Lamy L, Davis RE, Xiao W, Powell J, Maloney D, Thomas CJ, Moller P, Rosenwald A, Ott G, Muller-Hermelink HK, Savage K, Connors JM, Rimsza LM, Campo E, Jaffe ES, Delabie J, Smeland EB, Weisenburger DD, Chan WC, Gascoyne RD, Levens D, Staudt LM (2010) Cooperative epigenetic modulation by cancer amplicon genes. Cancer Cell 18(6):590–605
185. Kuppers R (2009) The biology of Hodgkin's lymphoma. Nat Rev Cancer 9(1):15–27
186. Gunawardana J, Chan FC, Telenius A, Woolcock B, Kridel R, Tan KL, Ben-Neriah S, Mottok A, Lim RS, Boyle M, Rogic S, Rimsza LM, Guiter C, Leroy K, Gaulard P, Haioun C, Marra MA, Savage KJ, Connors JM, Shah SP, Gascoyne RD, Steidl C (2014) Recurrent somatic mutations of PTPN1 in primary mediastinal B cell lymphoma and Hodgkin lymphoma. Nat Genet 46(4):329–335

Chapter 10
Chromosomal Translocations and Gene Rearrangements in Acute Lymphoblastic Leukaemia

Marc R. Mansour and A. Thomas Look

Contents

10.1 Introduction 190
10.2 Mechanisms of Chromosomal Translocations in ALL 192
10.3 Alterations in Ploidy in ALL 193
10.4 Dysregulated Expression of Structurally Intact Genes 194
10.4.1 Activation of MYC in ALL 194
10.4.2 Other Translocations Involving the IGH Locus 195
10.4.3 TAL1-Positive T-ALL 195
10.4.4 LMO1/LMO2 Driven T-ALL 196
10.4.5 *TLX1* and *TLX3* Genes in T-ALL 198
10.5 Chimeric Transcription Factor Genes 198
10.5.1 *ETV6-RUNX1 (TEL-AML1)* Fusion Gene in Early Pre-B ALL 198
10.5.2 *TCF3/E2A-PBX1* Fusion Gene in Pre-B-ALL 200
10.5.3 *TCF3-HLF* Fusion Gene in Pro-B-ALL 200
10.5.4 Translocations Involving the Mixed Lineage Leukaemia (*KMT2A/MLL*) Gene 201
10.6 Tyrosine Kinase Genes 202
10.6.1 Philadelphia-Chromosome Positive and the *BCR-ABL1* Gene in Early-B ALL 202

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10.6.2 Activation of CRLF2, IL7R and JAK2 in ALL..... 204
10.6.3 *NUP214-ABL1* Episomal Amplification in T-ALL..... 206
10.7 Future Directions..... 206
References..... 207

Abstract Acute lymphoblastic leukaemia (ALL) is a malignant clonal disorder arising from haematopoietic stem and progenitor cells, manifest as an overgrowth of lymphoid blast cells resembling early B- or T-cell progenitors. ALL occurs as a result of genetic, epigenetic and chromosomal aberrations that lead to unregulated cellular proliferation, a block in lymphocyte differentiation, aberrant cell survival and enhanced self-renewal. Chromosomal lesions can lead to loss of tumour suppressor genes or activation of structurally intact oncogenes, or create novel chimeric transcription factors or activated tyrosine kinases. Here we discuss the mechanisms of gene dysregulation in ALL, such as those occurring through translocation of oncogenes to immunoglobulin or T-cell receptor loci, often mediated by RAG-mediated double-strand breaks. Furthermore, we summarise recent findings from genome and transcriptome resequencing of ALLs that have implications for prognosis and targeted therapy.

Keywords Leukaemia • ALL • Aneuploidy • Translocation • RAG-2

10.1 Introduction

Acute lymphoblastic leukaemia (ALL) is a malignant clonal disorder arising from haematopoietic progenitors as a result of genetic, epigenetic and chromosomal aberrations that lead to unregulated cellular proliferation, a block in lymphocyte differentiation and enhanced self-renewal. What has become clear from gene expression profiling and next generation sequencing is that many of the aberrantly expressed oncogenes driving these phenotypes in ALL are transcription factors that play a carefully choreographed role in normal thymocyte development [1–4]. Furthermore, ALL cases can be clustered into groups that represent the maturation stage of a normal lymphoid counterpart, each cluster enriched in particular combinations of mutational lesions, with unique prognostic implications [3, 5, 6]. The marked difference in prognosis between paediatric (90 % 5-years overall survival, OS) and adult ALL (less than 50 % 5-years OS) [7–9], can be explained at least in part by the differing frequencies of chromosomal translocations and other genetic abnormalities that occur with age (Fig. 10.1). Most notably, older adults have a marked increase in Philadelphia-chromosome positive *BCR-ABL1 ALL* (Ph+ ALL) and lower frequency of *ETV6-RUNX1* ALL (Fig. 10.1) [10].

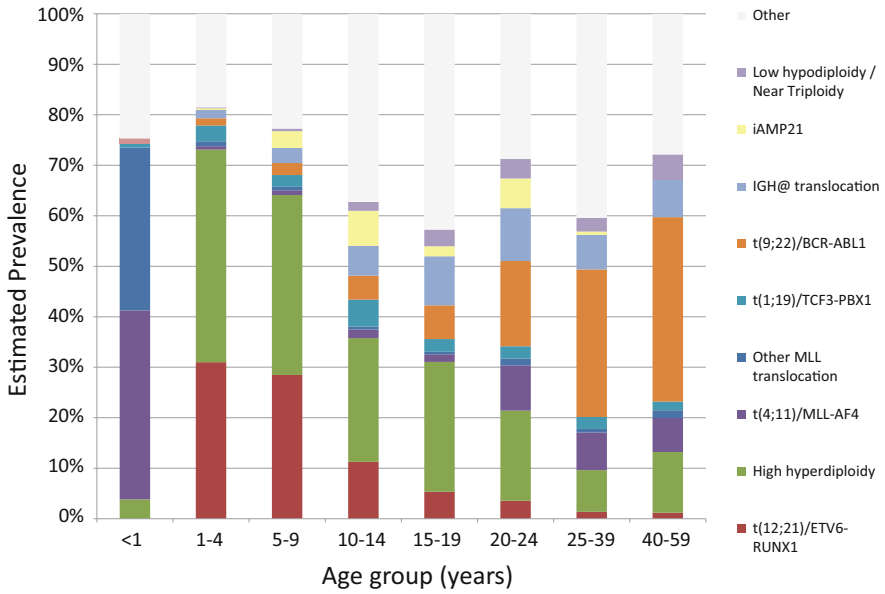

Fig. 10.1 Approximate age-associated frequencies of chromosomal abnormalities in B-ALL from a total cohort of 7113 patients from the UK MRC ALL trials. Patient ages were: <1 year n = 142, 1–4 years n = 3,128, 5–9 years n = 1,583, 10–14 years n = 915, 15–19 years n = 452, 20–24 years 169, 25–39 years n = 350, 40–59 years n = 374 (Data courtesy of Anthony Moorman and Christine Harrison, Leukaemia Research Cytogenetics Group (LRCG), Northern Institute for Cancer Research, Newcastle University)

Many oncogenes in ALL are activated through chromosomal translocation into the vicinity of tissue specific enhancers, such as to immunoglobulin heavy chain (*IGH*) enhancers in B-ALL, or T-cell receptor (*TCR*) enhancers in T-ALL, and are expressed as structurally intact proteins. The most common *TCR* genes involved in translocations are *TCRδ* (*TRD*) (67 %), followed by *TCR*β (*TRB*) (28 %) and *TCRα* (*TRA*) (5 %), with a variety of partner genes, the commonest partners being *LMO2*, *TAL1* and *TLX1* (Table 10.1) [11]. However, the majority of chromosomal translocations found in early B-ALL produce chimeric fusion proteins, with abnormal localisation or neomorphic function.

For many of the intact oncogenic transcription factors, transformation occurs in an anachronistic fashion: in other words, they are expressed at a developmental time point at which they are normally silent. *TAL1*, for instance, is normally only expressed up to the double-negative-2 (DN2) stage of thymic development, yet most *TAL1*-positive T-ALLs are arrested at the double-positive (DP) stage, meaning that TAL1 and its target genes are activated at a time point when they are normally not expressed [12–15].

Table 10.1 Frequency of cytogenetic translocations identified in T-ALL

Gene family	Gene name	Cytogenetic aberration	Frequency in T-ALL
bHLH family	*TAL1*	t(1;14)(p32;q11.2)	3 %
		t(1;7)(p32;q34)	3 %
		1p32 deletion	16 %–30 %
	TAL2	t(7;9)(q34;q32)	1 %
	LYL1	t(7;19)(q34;p13.2)	1 %
	OLIG2/BHLHB1	t(14;21)(q11.2;q22.1)	1 %
LMO family	*LMO1*	t(11;14)(p15.4;q11.2)	1 %
		t(7;11)(q34;p15.4)	1 %
	LMO2	t(11;14)(p13;q11.2)	6 %
		t(7;11)(q34;p13)	6 %
		11p13 deletion	3 %
	LMO3	t(7;12)(q34;p12.3)	<1 %
Homeobox family	*TLX1*(*HOX11*)	t(10;14)(q24.3;q11.2)	5–30 %
	TLX3(*HOX11L2*)	t(5;14)(q35;q32.3)	5–20 %
	HOXA	inv(7)(p15.2q34)	3 %
		t(7;7)(p15.2;q34)	3 %
	HOXA (PICALM-MLLT10)[a]	t(10;11)(p12.3;q14.2)	5–10 %
	HOXA (KMT2A-MLLT1)[a]	t(11;19)(q23.3;p13.3)	1 %
	HOXA (SET-NUP214)[a]	9q34 deletion inv(14)(q11.2q13)	3 %
	NKX2-1	inv(14)(q13.3q32.3) t(7;14)(q34;q13.3)	5 %
	NKX2-2	t(14;20)(q11.2;p11.2)	1 %
Oncogene	*NOTCH1*	t(7;9)(q34;q34)	<1 %
	MYB	t(6;7)(q23;q34)	3 %
	MYC	t(8;14)(q24.2;q11.2)	2 %
Cell cycle	*CDKN2A/B*	Deletion 9p21.3	>70 %
	CCND2	t(7;12)(q34;p13.3)	1 %
	RB1	t(12;14)(p13;q14.2)	4 %
		Deletion 13q14.2	
Signal transduction	*NUP214-ABL1*	Episomal amplification of 9q34	4 %

[a]These translocations have been show to directly upregulate the *HOXA* cluster and so are included in the *HOXA* category here. Frequencies are approximate and vary in adult and paediatric ALL

10.2 Mechanisms of Chromosomal Translocations in ALL

To recognise an almost endless diversity of antigens, B- and T-cells rearrange their immunoglobulin and TCR loci through V(D)J recombination, a process mediated by *recombination activating genes 1* and *2* (*RAG1*/*RAG2*) [16, 17]. RAG proteins are endonucleases that bind V(D)J segments through recognition of the recombination signal sequence (RSS): a palindromic heptamer sequence (consensus motif:

5′-CACAGTG-3′) separated from an AT-rich nanomer sequence (consensus motif: 5′-ACAAAAACC-3′) by a 12 or 23 nucleotide spacer [18, 19]. However, RAG proteins do not always faithfully obey the so-called 12/23 rule, and can erroneously create double strand breaks (DSB) at cryptic RSS sites (cRSS). These DNA ends are repaired by non-homologous end joining (NHEJ) and, as is the case for ALL, can adjoin with another DSB at a separate site to form chromosomal translocations, interstitial deletions or dicentric chromosomes. It has been shown that the chance of any two particular sites pairing is related to chromosome organisation, with DSBs sharing a topological nuclear domain most likely to pair [20, 21].

Translocations resulting from two cRSS DSBs have been termed 'Type I', and are exemplified by translocations involving *TAL1* and *LMO2* [11, 22, 23]. 'Type 2' translocations involve a DSB at a site that does not resemble a cRSS (i.e., likely not mediated by RAG), such as occur at the *TLX1* locus, and one RAG-mediated DSB at a TCR gene at a canonical RSS [11, 22, 23]. Thus, in addition to DSB generated by ectopic insults such as ionising radiation and mutagens [24], developing lymphocytes are under constant threat of DSBs initiated through the developmentally timed expression of RAG proteins. Consequently, the high frequency of microdeletions, focal amplifications, and chromosomal translocations identified in ALL may be attributable to RAG-mediated DSBs at RSS and cRSS sites [25]. It is also likely that at least some of the smaller indels and missense mutations found in ALL are the result of terminal deoxynucleotidyltransferase (TdT) activity at the site of a DSB, leading to the inclusion of non-template-derived nucleotides [25]. One further mechanism unique to ALL that can create somatic genomic lesions is reintegration of the DNA segments excised during V(D)J recombination (the episomal circles), although how wide a role this plays in leukaemogenesis is not yet clearly established [26].

One proposed model of leukaemogenesis is that translocations occur at a background level during normal lymphopoiesis; translocations involving oncogenes impart a survival and growth advantage to cells and impair terminal differentiation, prior to the acquisition of other secondary mutations that together drive overt leukaemic transformation [27–32]. However, it has also been shown that somatic mutation of an oncogene can occur prior to acquisition of chromosomal translocations; whether the chronology of genetic lesions impacts the behaviour of the tumour cells and response to therapy is not currently known [33].

10.3 Alterations in Ploidy in ALL

Some of the first examples of using chromosomal aberrations as prognostic markers in cancer come from the study of ploidy in ALL [34–37]. ALL patients with hypodiploidy (≤44 chromosomes) have a poor prognosis, whereas those with hyperdiploidy (>50 chromosomes) tend to have a favourable response to chemotherapy, particularly if this involves trisomy of chromosomes 4, 10, and 17 [38–41]. The exception is near tetraploidy (82–94 chromosomes), which is often associated with T-ALL and does not confer a survival benefit in ALL [42].

Hyperdiploidy is found in approximately one-quarter of paediatric patients, particularly those aged 2–10 years old, is less common in adult ALL, and is associated with an early pre-B immunophenotype and low white blood cell count (WCC) [43–45]. Similar to acute myeloid leukaemia (AML), nearly 25 % of hyperdiploid B-ALL cases have mutations in *FMS-related tyrosine kinase-3* (*FLT3*). However, the classic AML-associated *FLT3*-internal tandem duplication (ITD) is rare in ALL, with the majority of mutations occurring as point mutations that activate the tyrosine kinase domain (TKD), or occasionally as in-frame deletions of the juxtamembrane domain [46, 47].

Hypodiploid ALL can be subdivided into high hypodiploid (42–45 chromosomes), low hypodiploid (33–39 chromosomes) and near haploid (23–29 chromosomes), with the worse prognosis associated with the lowest number of chromosomes [48]. Recent studies have shed light on the molecular pathogenesis of these leukaemic subtypes. Near haploid ALL is associated with activating mutations of tyrosine kinase genes and those involved in RAS signalling (71 %), whereas over 90 % of low hypodiploid ALL patients have mutations in *TP53*, many of which are germline in origin and usually accompanied by loss of the second *TP53* allele due to monosomy 17, a feature likely responsible for the very poor outcome in this leukaemic subtype [48–50].

Interestingly, one chromosome that is almost never lost, even in near haploid ALL, is chromosome 21. Consistent with the concept that critical oncogenes reside on chromosome 21, Down syndrome (DS) (trisomy 21) is associated with a 20-fold increased risk of ALL [51]. Recent elegant studies in mice implicate the *HMGN1* gene on chromosome 21q22, encoding a nucleosome remodelling protein, as the critical triplicated gene in both trisomy 21 and somatic iAMP21 [52].

10.4 Dysregulated Expression of Structurally Intact Genes

10.4.1 Activation of MYC in ALL

The *MYC* oncogene is frequently activated in both T- and B-lineage ALL, through distinct mechanisms. The t(8;14)(q24.2;q32.3) translocation, classically found in the majority of Burkitt lymphomas and occasionally in B-ALLs that express cell surface immunoglobulin [53, 54], places MYC under the control of *IGH* locus enhancers leading to its constitutive activation in B-cells. The rarer variant translocations, t(2;8)(p12;q24.2) and t(8;22)(q24.2;q11.2), involve juxtaposition of MYC to the κ or λ light-chain genes on chromosomes 2 and 22 respectively.

The majority of cases of T-ALL, as well as cell lines established in culture, show activation and dependency on MYC. Only very rarely is this the result of chromosomal translocation. A more common mechanism relates to the finding that over 50 % of patients have activating mutations in *NOTCH1* [30, 55, 56] that directly activates c-MYC through transcriptional binding of Notch-RBPJk at the *MYC* promoter [57–59]. MYC can be further stabilised post-translationally as a result of

loss-of-function of FBXW7 E3-ubiquitin ligase activity, occurring through its mutational inactivation or *miR-223*-mediated suppression [12, 60–63].

Despite the extensive work on the role of MYC in cancer over the last decades, the mechanism through which it exerts its oncogenicity is only now being understood. A recent discovery of particular interest is that MYC is a global amplifier of gene expression, a finding that had not been appreciated previously because most gene expression arrays platforms are normalised based on global expression levels [64, 65]. Thus, rather than having a specific set of MYC 'target genes', MYC accumulates at the promoter of active genes, amplifying the existing gene expression programmes, a mechanism termed 'transcriptional amplification'. MYC mediates promoter pause release and transcriptional elongation through the recruitment of the elongation factor pTEFb, which in turn phosphorylates and activates the C-terminal domain (CTD) of RNA Polymerase II [65–67].

10.4.2 Other Translocations Involving the IGH Locus

Analysis of a cohort of 3,269 ALL patients identified *IGH* translocations not involving *MYC* in approximately 5 % of B- and T-lineage ALL patients [68]. Interestingly, there was an enrichment of *IGH* aberrations in adolescent patients and those with DS-ALL (16 %). The most commonly identified partner genes were *CRLF2* (22 %), the *CEBP* gene family (11 %) and *ID4* (7 %). B-ALL patients with *IGH* translocations have an inferior prognosis, which is independent of involvement of CRLF2 or DS-ALL. Rare *IGH* translocation partners of note include *EPOR* and *IL3* in B-ALL. The latter, resulting from the t(5;14)(q31.1;q32.3), although extremely rare, is a distinct entity in the World Health Organization (WHO) classification of B-ALL, as it carries a poor prognosis and has a characteristic eosinophilia. *IGH* translocations are also found in 3–20 % of T-ALL cases, and are more common in TCRγδ than TCRαβ T-ALL, with partner genes including *TAL1* and *TCR*α/δ (*TRA*/*D*) [68, 69].

It is notable that the majority of ALL-associated *IGH* translocation partners are not found in mature B-cell malignancies, suggesting their oncogenic potential is dependent on the stage of differentiation arrest [70]. For instance, *ID4* is an oncogene in ALL but has a tumour-suppressor role in chronic lymphocytic leukaemia [71].

10.4.3 TAL1-Positive T-ALL

TAL1 is a class II bHLH transcription factor that is required for early definitive haematopoiesis, and is expressed by early thymocytes until the DN2 stage of T-cell development [13–15, 72]. Over 50 % of T-ALL patients aberrantly overexpress *TAL1*, either through translocation to the *TRA* locus from the t(1;14)(p32;q11.2) (in 3 % of T-ALLs), or more commonly through an ~80-kb deletion on 1p33 that fuses the regulatory elements of the ubiquitously expressed gene "*SCL*-interrupting

locus" (*STIL*) to TAL1 [3, 73–77]. We recently discovered that approximately 5 % of T-ALL cases harbour somatic 2–18 bp insertions in a precise non-coding site 8 kb upstream of the *TAL1* transcriptional start site, which introduce novel MYB binding motifs that recruit MYB together with the TAL1 complex to form a large super-enhancer that drives monoallelic *TAL1* expression [78]. This established MYB as the newest member of the TAL1 complex of transcriptional regulatory proteins, given the vast majority of TAL1 sites are co-occupied by MYB genome-wide, and suggests a role for MYB in the formation of super-enhancers that define cell state during the normal development of haematopoietic stem and progenitor cells.

To bind DNA, TAL1 forms obligate heterodimers with a class I bHLH proteins such as HEB and TCF3/E2A, and nucleates a transcriptional complex that includes the GATA-1 or -3 (in erythroid precursors and thymocytes respectively), LIM domain only 1/2 (LMO1/LMO2), RUNX1 and MYB proteins [79, 80]. Although the TAL1 complex can mediate transcriptional repression through recruitment of repressors such SIN3A and HDAC1/2, in T-ALL it binds the histone acetyltransferase CREBBP to act as a potent activator of gene expression [81–83]. ChIP-seq technology has shown that this complex activates the majority of its critical target genes through binding to their enhancers, including important T-ALL oncogenes, such as *NKX3-1*, *MYB*, *STAT5*, and *TRIB2*. Notably, the complex itself forms a positive interconnected auto-regulatory loop, whereby the core components, TAL1, GATA3, RUNX1, and MYB positively regulate their own enhancers (Fig. 10.2) [78, 83]. The TAL1 complex activates other genes, such as miR-223, through promoter binding [12, 83, 84]. *miR-223* is the most abundantly expressed miRNA in TAL1 positive T-ALLs, where it acts to suppress the FBXW7 tumour suppressor, which functions in phosphorylation-dependent ubiquitination, and whose down-regulation stabilises MYC, MYB, NOTCH, MCL1 and MTOR oncoproteins (Fig. 10.2) [12, 85].

10.4.4 LMO1/LMO2 Driven T-ALL

The LIM-only domain gene, *LMO2* (formerly *RBTN2*/*TTG2*) is aberrantly expressed in approximately 10 % of T-ALL cases, most commonly through t(11;14)(p13;q11.2), or more rarely t(7;11)(q34;p13), involving the *TRD*/*TCRδ* or *TRB*/*TCRβ* loci respectively [86–88]. The mechanism of aberrant *LMO2* activation is distinct from other translocation partners of the *TCR* loci, in that loss of the *LMO2* negative regulatory element (NRE), rather than juxtaposition to the *TCR* enhancer, is the main driver of *LMO2* overexpression [89, 90]. This is supported by the finding that approximately 4 % of T-ALL patients have del(11)(p12p13), that drives *LMO2* expression through deletion of the NRE [91]. The oncogenic potential of LMO2 activation in human T-ALL is best exemplified by four cases of T-ALL that have occurred secondary to gene therapy for severe combined immunodeficiency, where the lentiviral insertion occurred in the vicinity of *LMO2* oncogene leading to its upregulation by the enhancer effect of the viral long-terminal repeats (LTR) [31, 92–95].

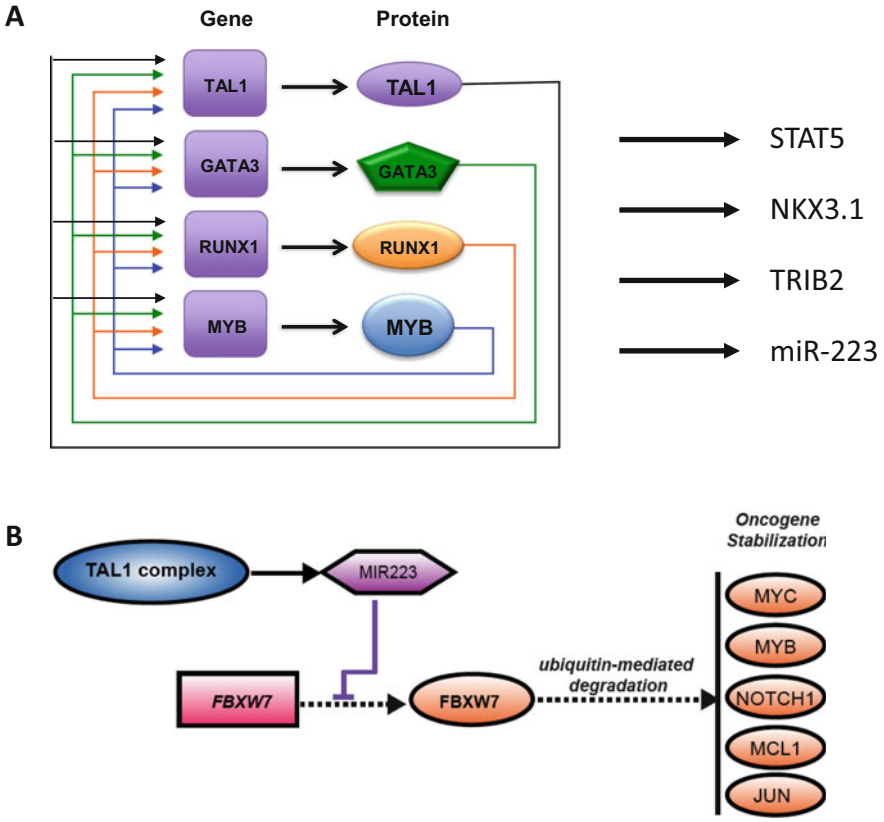

Fig. 10.2 (**a**) Schematic of the positive interconnected autoregulatory loop whereby each core component of the TAL1 complex, including TAL1 itself, GATA3, RUNX1 and MYB, regulate their own enhancers, thereby reinforcing and stabilising the TAL1-regulated oncogenic programme in T-ALL [83]. (**b**) Schematic showing the proposed model by which the TAL1 complex activates miR-223 through binding to its promoter, which in turn suppresses FBXW7 expression, thereby stabilising the oncogenic substrates MYC, MYB, NOTCH1, MCL1 and JUN [12]

LMO proteins cannot directly bind DNA, but act as a bridging molecule in a transcriptional complex that includes the GATA, LBD1 and TAL1 proteins [96–98]. By gene expression profiling, *LMO2*-positive T-ALL cases cluster in two distinct groups: LMO1/LMO2 translocated cases closely resemble the TAL1-positive subgroup, whereas a second group exists that is highly enriched for a signature of normal early T-cell progenitors characterised by high expression of *MYCN*, *LYL1* and *HHEX* [3, 99]. Murine models of *LMO1*/*LMO2* driven T-ALL likely represent this latter group given that they highly express these genes, of which *HHEX* appears to be particularly important because it is a direct target of LMO2 that mediates thymocyte self-renewal and is required for leukaemogenesis [100–106]. In mice, *TAL1* accelerates T-ALL onset in combination with *LMO2*, consistent with the finding that leukaemogenesis is mediated through a transcriptional complex containing both proteins [98]. The long latency (12–18 months) of T-ALL in *LMO2* transgenic

mouse lines suggests that secondary events are required for transformation [102]. Indeed, the majority of *LMO1*/*LMO2* derived murine leukaemias, and gene therapy induced T-ALL cases, harbour somatically acquired activating mutations of *NOTCH1* [31, 93, 107].

10.4.5 TLX1 *and* TLX3 *Genes in T-ALL*

The homeodomain gene *TLX1* (formerly *HOX11*), is aberrantly expressed in 5 % of paediatric and as many as 30 % of adult T-ALL cases [2, 108–110]. Approximately one-half of these cases harbour the classic t(10;14)(q24.3;q11.2), with the majority of other cases overexpressing *TLX1* monoallelically with no known *TLX1* translocation [109, 110], suggesting an as yet undiscovered *cis*-acting lesion. *TLX1* cases are arrested at an early cortical thymocyte stage of development characterised by high expression of CD4+CD8+CD1a+: such cases are associated with a very high frequency of *NOTCH1* mutations and a very good prognosis [108, 111]. Mouse models of *TLX1*-driven T-ALL have shown that *TLX1* drives aneuploidy in T-ALL cells and collaborates with mutations and deletions of the *BCL11B* transcription factor [112].

TLX3 (formerly *HOX11L2*) is overexpressed in about one quarter of children but only 5 % of adults with T-ALL. *TLX3* is most frequently activated through the cryptic t(5;14)(q35;q32.3) [2, 113–116] which juxtaposes the *BCL11B* regulatory elements from chromosome 14, highly active during thymocyte development, to the *TLX3* locus [117, 118]. Thus, haploinsufficiency for the *BCL11B* tumour suppressor is also a consequence of this translocation. Variant translocations have also been described including *TLX3* translocated to *CDK6* in the t(5;7)(q35;q21.2) [119]. The prognostic significance of *TLX3* overexpression is controversial: it is likely that the poor prognosis initially reported in this subgroup is only applicable to those patients who also express the *NUP214-ABL1* fusion gene [114, 120, 121].

10.5 Chimeric Transcription Factor Genes

10.5.1 ETV6-RUNX1 (TEL-AML1) *Fusion Gene in Early Pre-B ALL*

Fusion of the *ETV6* (previously *TEL*) and *RUNX1* (previously *AML1*) genes resulting from t(12;21)(p13.2;q22.3) is the most common structural chromosomal alteration in paediatric early B-ALL, present in approximately 25 % of cases, although rare (~3 %) in adult early B-ALL [40, 122–125]. It is cryptic, but readily detectable by a variety of molecular and cytogenetic techniques, and frequently shows loss of the normal *ETV6* allele, suggesting that complete loss of normal *ETV6* contributes to the oncogenic effect of ETV6-RUNX1 fusion protein [122, 125–129].

RUNX1 was originally identified in the AML-associated t(8;21)/*RUNX1-RUNX1T1* (formerly *AML1-ETO*) fusion transcript [130], a translocation that

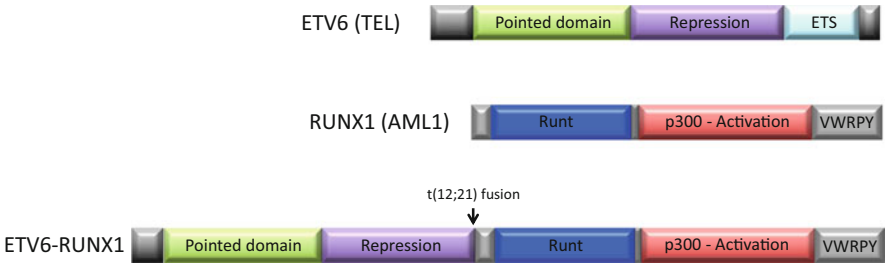

Fig. 10.3 Schematic depicting the *ETV6* (*TEL*) and *RUNX1* (*AML1*) genes, together with the highly recurrent *ETV6-RUNX1* fusion gene identified in B-ALL secondary to the t(12;21) (p13.2;q22.3). The ETS DNA-binding domain, central repression domain and pointed oligomerisation domains are shown for ETV6. For *RUNX1* (*AML1*), the activation domain shown also includes the p300 HAT interacting domain and the mSIN3A interaction domain. The VWRPY C-terminal sequence binds to NCOR and the Groucho homologs TLE1-4, which mediate transcriptional repression

involves loss of the C-terminal regulatory sequences of *RUNX1*, but retention of the amino-terminal RUNT domain [131]. In this regard, ETV6-RUNX1 differs from RUNX1-RUNX1T1 in that the chimeric protein retains all functional domains of RUNX1 (Fig. 10.3) [123, 132, 133]. Although this includes the transactivation domain, including the p300 interaction domain, reporter assays support a model in which ETV6-RUNX1 antagonises the transactivation potential of wild-type RUNX1, mediated through the recruitment of NCOR, and/or the Groucho repressors TLE1-4 that bind the C-terminal VWRPY motif of RUNX1 [132, 134, 135].

Transgenic mice expressing *ETV6-RUNX1* do not, however, develop overt leukaemia [136, 137]. Work from the laboratory of Mel Greaves has shown that *ETV6-RUNX1* is detectable in approximately 1 % of neonates, of which 1 % will go on to develop overt B-ALL [138]. Prospective analysis of such carriers has shown that *ETV6-RUNX1* expands a CD34+CD38-CD19+ pool of early B-cells with self-renewal potential that can remain stable over many years [28]. This would suggest a model in which *ETV6-RUNX1* arises prenatally as a first hit and that secondary collaborating mutations, such as loss of the wild-type *ETV6* allele [126], are required to transform cells to frank leukaemia. One intriguing theory is that an exaggerated response to viral infection preferentially expands the pre-leukaemic clone through activation of TGFβ signalling and is a key mediator of transformation [137].

Presence of *ETV6-RUNX1* is associated exclusively with B-lineage ALL, and a distinct subgroup of young patients (1–10 years) with non-hyperdiploid ALL and an excellent prognosis, with survival rates exceeding 90 % [40, 41, 122, 139]. Whether the favourable response to chemotherapy directly relates to the biology of the *ETV6-RUNX1* fusion transcript is unclear because it is not an independent predictor of prognosis when age and white blood cell count are also taken into account, particularly on current protocols involving intensive asparagine depletion [139]. However, the ETV6-RUNX1 fusion protein has also been shown to directly repress the multidrug resistance gene (*MDR1*) [140], and is associated with low expression of genes involved in purine metabolism [141] and, thus may sensitise cells to drugs such as methotrexate and mercaptopurine, that inhibit *de novo* purine synthesis.

10.5.2 TCF3/E2A-PBX1 *Fusion Gene in Pre-B-ALL*

The TCF3-PBX1 fusion protein resulting from t(1;19)(q23;p13.3) occurs in approximately 5 % of all early B-ALLs, and all cases expressing *TCF3-PBX1* also express cytoplasmic immunoglobulin (*TCF3-PBX1* is found in 20–25 % of such cases) [40, 142, 143]. Although the presence of *TCF3-PBX1* was previously associated with a poor prognosis, its presence has no prognostic significance on more recent paediatric and adult ALL treatment protocols [143–148]. The oncogenic fusion protein consists of exons 1–16 of the 5′ portion of *TCF3* from chromosome 19, encoding the TAD (AD1 and AD2), but not its bHLH DNA-binding domain, fused to the 3′ exons 4–9 of *PBX1* from chromosome 1 encoding the homeobox DNA-binding domain [149–151]. The reciprocal 5′ *PBX1* – 3′ *TCF3*, if present, is not transcribed. Structure-function experiments of the TCF3-PBX fusion protein, have shown that the AD1 and AD2 domains of TCF3 are required for cell transformation of NIH-3T3 fibroblasts, while the homeodomain of PBX1 is dispensable, since a mutant TCF3-PBX1 construct lacking the homeodomain is still able to transform NIH-3T3 cells and form lymphomas in mice [152]. Later studies have shown that a small region downstream of the PBX1 homeodomain known as the Hox cooperativity motif (HCM) is required for transformation, most likely due to its interaction with HOX proteins [153].

One of the limitations of mouse modelling of TCF3-PBX1 in mice has been that its ectopic expression results in either T-cell lymphomas or AML, whereas the translocations in humans are confined to cytoplasmic immunoglobulin expressing pre-B-lineage ALL [154, 155]. Even when *TCF3-PBX1* is driven from *IGH* regulatory sequences specific to B-cells, mice still develop lymphomas of T-cell origin, probably because TCF3-PBX1 has been shown to initiate apoptosis in murine B-cell lineage cells in a manner that is TP53-independent and that can be overcome by BCL2 overexpression [156]. Thus, identifying the critical genes downstream of TCF3-PBX1 in pre-B-ALL has proven challenging, and one that hopefully will be answered with newly available technologies, such as ChIP-seq, that can identify the direct targets of endogenous TCF3-PBX1 in primary human pre-B-ALL samples.

10.5.3 TCF3-HLF *Fusion Gene in Pro-B-ALL*

The t(17;19)(q22;p13.3) resulting in the TCF3-HLF fusion protein is a rare balanced translocation occurring in less than 1 % of B-ALL cases, usually of the pro-B subtype. *TCF3-HLF* is characteristically associated with a low WCC ($<20 \times 10^9$/l), aberrant expression of CD33, disseminated intravascular coagulation and hypercalcemia (from high levels of parathyroid hormone-related peptide) at diagnosis, and a dismal response to chemotherapy, with all reported patients experiencing early relapse and succumbing to disease progression [40, 157–159]. Similar to TCF3-PBX1, the fusion protein retains the AD1 and AD2 transactivation domains of TCF3

but, in this case, is fused to the C-terminal bZIP DNA binding and dimerization domain of HLF [160, 161]. HLF is a bZIP transcription factor of the proline and acidic-rich (PAR) protein family, which, like many other bZIP proteins, can bind DNA either as a heterodimer or homodimer. A mutant construct encoding TCF3-HLF containing an extra coil of the leucine zipper domain, which cannot bind DNA as a heterodimer with the other PAR family members, is equally efficient in transforming NIH-3T3 cells, suggesting TCF3-HLF homodimers are responsible for tumourigenicity [162]. Notably, each of the AD1, AD2 and leucine zipper domains are also required for cell transformation of NIH 3T3 fibroblasts [162, 163].

TCF3–HLF can immortalise haematopoietic progenitor cells, occurring predominantly through an acquired resistance to apoptosis [164, 165]. Several downstream target genes have been implicated in this phenotype include *LMO2*, *BIRC5* (*survivin*), *SLUG* and *BCL2* [165–168]. From a clinical perspective, perhaps the most promising targeted agents that warrant accelerated testing for this devastating subtype of ALL are BCL2 inhibitors, such as ABT-199, given these are already in clinical trials and induce apoptosis of *TCF3–HLF* transformed cells *in vitro* [169].

*10.5.4 Translocations Involving the Mixed Lineage Leukaemia (***KMT2A/MLL***) Gene*

The *mixed lineage leukaemia gene*, *KMT2A* (formerly *MLL*), is located at 11q23.3, and has been identified as a translocation partner of over 80 other genes [170–173]. *KMT2A* translocations occur in many different subtypes of acute leukaemia, including AML, B-ALL and T-ALL. The two patient populations where *KMT2A* rearrangements are particularly recurrent are infantile leukaemia (80 %) and AML secondary to chemotherapy (t-AML) (85 % after topoisomerase II inhibitor therapy) [40, 174–176].

In infantile leukaemia (children <1 year of age, including B-ALL, AML or mixed lineage subtypes), the majority of patients harbour a *KMT2A* gene translocation, often the t(4;11)(q21.3;q23.3) producing the *KMT2A-AFF1* (formerly *AF4*) fusion gene [177]. The presence of *KMT2A* translocations detectable in the neonatal blood spots (Guthrie cards) of infants who have developed leukaemia strongly supports an *in utero* origin for the majority of infantile *KMT2A* leukaemias [27]. Furthermore, concordance rates of infantile leukaemia in identical twins have been estimated to be as high as 100 %, with the majority of twin pairs exhibiting identical immunoglobulin gene rearrangements, indicating that the tumours occurred *in utero* and were transmitted from one twin to the other through a shared placental circulation [178, 179].

KMT2A, a homologue of the *Drosophila* trithorax protein, has Histone 3 Lysine 4 (H3K4) methyltransferase activity provided by its C-terminal domain and forms a large chromatin remodelling complex together with Swi/Snf family members, CREBBP and HDACs, that together are required for primitive and definitive haematopoiesis, predominantly mediated through activation of *HOX* genes [180, 181]. The

majority of *KMT2A* translocations occur in a defined 8.3 kb breakpoint cluster region (BCR) between exons 5 and 11, retaining the N-terminal AT-hook domain, which binds DNA in a non-specific manner, and the CxxC DNA-binding domain, which can only bind unmethylated DNA [182]. The C-terminal portion of the chimeric protein is provided by the partner gene. The most common translocation partner in early B-ALL is *AF4*, derived from the t(4;11)(q21.3;q23.3), followed by *MLLT1*/*ENL*, derived from the t(11;19)(q23.3;p13.3) and finally *MLLT3*/*AF9*, derived from the t(9;11)(p21.3;q23.3) [183]. Together these three genes account for nearly 90 % of the KMT2A partners found in early B-ALL [184]. In T-ALL, the most common *KMT2A* partner gene by far is *MLLT1*, derived from the t(11;19)(q23.3;p13.3).

Many of the mechanistic insights into KMT2A oncogenicity have come from the study of murine models of *KMT2A*-induced leukaemia, including *KMT2A-MLLT3* chimeric mice [185], and haematopoietic stem cell (HSC) retroviral transduction experiments with *KMT2A-MLLT1*, *KMT2A-ELL*, *KMT2A-MLLT3* and *KMT2A-CREBBP* fusion genes, all of which induce leukaemia with varying latencies [186–188]. Gene expression profiling strongly implicates the HOX gene cluster, particularly *HOXA9* and *HOXA10*, and the HOX regulator, *MEIS1*, as critical downstream target genes for the malignant phenotype [189–193]. Recent data suggests that *KMT2A* fusion genes dysregulate target genes through chromatin remodelling by recruiting the histone methyltransferase DOT1L, which specifically methylates lysine-79 of histone H3 (H3K79) [194]. H3K79 methylation marks are enriched at KMT2A target genes, including the HOXA cluster, and such histone modifications are associated with actively transcribed genes, suggesting that DOT1L facilitates the oncogenic KMT2A transcription programme [194–196].

The prognostic significance of *KMT2A* rearranged leukaemias depends on the patient age, leukaemia subtype and the partner gene. *KMT2A* translocations carry a poor prognosis in infantile leukaemia, with only 40–50 % of children cured of their disease, even with the addition of allogeneic transplant in first remission [177, 197, 198]. Patients with *KMT2A* translocations and t-AML also fair extremely badly [199]. All age groups of ALL patient harbouring the t(4;11) have an inferior prognosis, although, due to their rarity, the relevance of the other partner genes is less well-defined [7, 10]. In T-ALL, the most common *KMT2A* translocation, t(11;19), is associated with a favourable outcome [200].

10.6 Tyrosine Kinase Genes

10.6.1 Philadelphia-Chromosome Positive and the BCR-ABL1 *Gene in Early-B ALL*

The discovery of the Philadelphia chromosome (Ph+) in chronic myeloid leukaemia (CML) by Peter Nowell and David Hungerford in 1960, was the first ever chromosomal lesion identified in cancer [201]. Janet Rowley subsequently reported that the

Ph chromosome arises from a translocation involving chromosome 9 and 22, later shown to generate a fusion gene involving the 5′ region of *BCR* fused to the 3′ sequences of *ABL1* [202, 203]. In early B-ALL, the frequency of t(9;22)(q34.1;q11.2) is very much dependent of patient age, present in only 2 % of children with early B-ALL, but steadily increasing in frequency to 40 % of early B-ALLs in adults over the age of 40 years [40, 145, 204]. Cases of Ph+ T-ALL, occurring either *de novo* or as a presentation of blast crisis of CML, have also been described in the literature, but are exceedingly rare [205].

On 9q34.1, breakpoints are scattered over a 200 kb region within the first intron of the *ABL1* proto-oncogene, between the alternative exons 1a and 1b, and upstream of the tyrosine kinase domain [206–208]. On chromosome 22, breakpoints occur in two regions of BCR. The Major-breakpoint cluster region (M-BCR), as seen in CML and approximately one-third of early B-ALL cases, involves a 5.8 kb region between exons 12 and 16, giving rise to a 210 KDa chimeric protein (p210). The minor breakpoint cluster region (m-BCR), occurring solely in Ph+ ALL, involves a 35 kb region between exons 1 and 2, resulting in a 190 KDa chimeric protein (p190) [209]. Rarely, a breakpoint occurs between exons 19 and 20 (“micro”-BCR breakpoint) to create a 230 KDa protein (p230). Expression of the p190 or p210 chimeric proteins by retroviral infection of murine marrow results in either a CML-like disease, or immature B-lymphoid leukaemia, depending on whether or not the donor marrow was harvested after 5-fluorouracil treatment, respectively [210–214]. Although both p190 and p210 forms of *BCR-ABL1* produce disease with a similar immunophenotype, the B-lymphoid neoplasms are significantly more aggressive with p190, consistent with its increased kinase activity *in vitro* [214].

The mechanism through which the BCR-ABL1 fusion protein exerts its oncogenicity has been extensively studied [reviewed in [215]]. An alteration in structure of the chimeric protein leads to increased ABL1 kinase activity due to loss of autoregulation by its SH3 domain, mediated in part through loss of the N-terminal myristate group of ABL1, and in part through the coiled-coil oligomerisation domain juxtaposed from the N-terminus of BCR [216–218]. Furthermore, ABL1 becomes strongly autophosphorylated, and is no longer confined to the nucleus, but instead can interact with alternative substrates in the cytoplasmic compartment, mediating aberrant pathway activation [218–220]. For instance, in the cytoplasm, BCR-ABL1 interacts with and activates MAP3K1 (previously MEK), which acts upstream of the extracellular signal regulated kinase (ERK), Jun N-terminal kinase (JNK), and NF-kB signalling pathways [221, 222]. Transformation by BCR-ABL1 also involves activation of RAS and PI-3 kinase (PI3K) pathways, CBL and CRKL, JAK-STAT, NFKB, and SRC, as well as upregulation of MYC [223–231]. JAK2-independent phosphorylation of STAT5 is a critical step in leukaemogenesis, given that dominant-negative STAT5 mutants markedly impair transformation potential of BCR-ABL [232]. BCR-ABL1 also collaborates with loss of IKZF1, as over 80 % of Ph+ B-ALL cases (but not chronic phase CML cases) harbour intragenic deletions of the IKZF1 loci, and are associated with a poor prognosis [233].

Until recently, Ph+ ALL has been associated with a poor prognosis across all age groups [234–236]. Proposed mechanisms of resistance to standard chemotherapy

include BCR-ABL1-mediated upregulation of the antiapoptotic genes *BCLXL* and *MCL1*, induction of multi-drug resistance (MDR) genes, such as *ABCB1* and *ABCC1*, and aberrant TP53 localisation after DNA-damage [237–239]. Although tyrosine kinase inhibitors (TKIs) such as imatinib are able to induce complete and sustained responses in chronic phase CML, their efficacy is limited when used as single agents in Ph+ ALL [240, 241]. Thus, similar to blast crisis CML, TK inhibitors need to be given in combination with standard chemotherapy in Ph+ ALL, consistent with the notion that other secondary genomic lesions contribute significantly to the progression to B-ALL. The combination of TKIs with chemotherapy has been very encouraging, with 3 year EFS rates as high as 80 % in children [242]. There is some expectation that survival rates will be further improved with the addition of the second generation TKI, dasatinib, which has considerably more activity than imatinib *in vitro* and also inhibits the SRC-family kinases [243, 244]. Dasatinib has been curative in some cases of Ph+ ALL even when given as monotherapy, and trials are underway exploring its efficacy and tolerability in combination with standard ALL protocols [215, 245, 246]. Whether the addition of TKIs to front-line chemotherapy obviates the need for allogeneic transplant in first remission is currently an issue of debate and on-going study [215].

10.6.2 Activation of CRLF2, IL7R and JAK2 in ALL

The JAK-STAT pathway is essential for the establishment of normal lymphopoiesis, as exemplified by the development of severe combined immunodeficiency (SCID) when key components of this pathway, such as the interleukin-*2* receptor common gamma-chain (*IL2RG*), *IL7* receptor alpha (*IL7RA*), *JAK3* or *RAG* genes are inactivated through inherited genetic lesions [247–249]. During normal lymphoid development, IL7R heterodimerises with either IL2R, or CRLF2, to form cell surface receptor complexes that respond to ligand stimulation with IL7 and thymic stromal lymphopoietin (TSLP) respectively, activating the JAK family, STAT5 and PI3K signalling (Fig. 10.4).

It has recently been appreciated that constitutive, ligand-independent activation of this pathway occurring through a variety of different genomic lesions, plays a prominent role in the pathogenesis of both B- and T-ALL. For instance, inactivating mutations or deletions of SH2B3 (encoding LNK, a negative regulator of JAK2), activating mutations in STAT5B through the N642H missense mutation (6 % of T-ALL cases) and, rarely, the F232C missense mutation in *CRLF2*, have all been recently discovered [250–252]. In 10 % of paediatric B-ALL cases, and over 50 % of Down Syndrome (DS) related B-ALL, *CRLF2* is activated through an intrachromosomal deletion of the pseudoautosomal region 1 (PAR1) of Xp22.3/Yp11.3 that juxtaposes the coding region of *CRLF2* to the regulatory elements of the neighbouring P2RY8, encoding a purinergic receptor highly expressed in ALL cells [51, 252, 253]. Interestingly, nearly half of DS-ALL patients with aberrant *CRLF2* expression in early B-ALL also have activating mutations of a JAK family member,

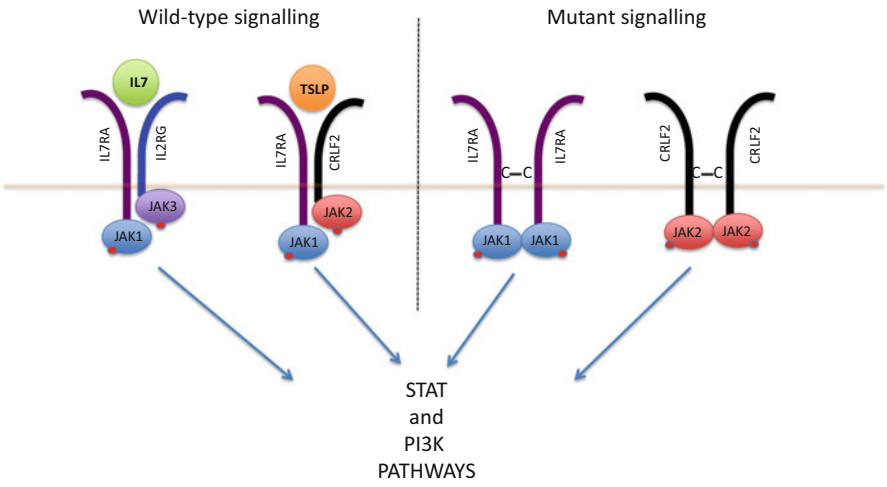

Fig. 10.4 Schematic of wild-type and mutant IL7R-JAK-STAT signalling. In normal B- and T-cell development, IL7 binds to an IL7RA-IL2RG heterodimeric complex, leading to phosphorylation and cross-phosphorylation of JAK1 and JAK3, which activates PI3K and STAT (predominantly STAT5) signalling. In Th2 T Cells, dendritic cells, macrophages and mast cells, thymic stromal lymphopoietin (TSLP) binds to CRLF2-IL7RA heterodimers to activate similar pathways. In B- and T-ALL, IL7RA is somatically mutated, and the majority of mutations introduce novel cysteines in the transmembrane domain, which create disulphide bonds with neighbouring mutant IL7RA receptors, to form constitutively active IL7RA homodimers, which activate JAK1-STAT5 signalling in a ligand independent manner. Recently, we have shown that treatment with N-acetylcysteine is effective in reducing the disulphide bonds and inducing apoptosis in ALLs addicted to constitutive IL7-JAK-STAT5 signalling [254]. The F232C mutation of CRLF2 found in early B-ALL activates JAK-STAT signalling in the same manner. It should also be noted that this pathway is activated by several other mechanisms in ALL, including by mutations of JAK1, 2 and 3, STAT5, and LNK, as well as CRLF2 over-expression, occurring through interstitial deletion or chromosomal translocation

most frequently *JAK2*, consistent with the finding that ectopic expression of *P2RY8-CRLF2* is insufficient to transform Ba/F3 cells to cytokine independence unless co-expressed with mutant *JAK2* [253]. Although initial studies suggested a poor prognosis in patients with high *CRLF2* expression, this remains controversial, being highly influenced by the individual treatment protocol and presence of other genetic lesions such as *IKZF1* deletions [252, 255–258].

Somatically acquired activating *IL7R* mutations occur in approximately 10 % of T-ALL, and 1 % of B-ALL cases, the vast majority of which are short in-frame insertions in exon 6 resulting in the introduction of a novel cysteine just extracellular to the transmembrane domain [4, 259, 260]. Similar to the F232C mutation of CRLF2, these unpaired cysteine residues lead to inter-molecular disulphide bond formation leading to ligand-independent receptor homodimerisation, and constitutive JAK1 and STAT5 activation (Fig. 10.4) [4, 259, 260]. Given inhibition of JAK-STAT signalling results in apoptosis of *IL7R*-mutant cells, there has been much interest in using clinically available JAK inhibitors, such as ruxolitinib, in IL7R

mutant T-ALL cases [4, 259–261]. Indeed, ruxolitinib results in an improvement in survival in murine models of mutant IL7R-driven ALL, although the benefit is modest [262]. As an alternative approach, we recently demonstrated that N-acetylcysteine (NAC), a reducing agent widely and safely used for paracetamol (acetaminophen) poisoning, can reduce the inter-molecular disulphide bonds of mutant IL7R, disrupt the aberrant homodimer, and induce apoptosis of IL7R-mutant T-ALL cells both *in vitro* and *in vivo* at doses readily achievable in humans [254].

10.6.3 NUP214-ABL1 *Episomal Amplification in* T-ALL

When using FISH for *ABL1*, Graux and colleagues identified a subset of T-ALL patient samples where there was clear evidence of multiple extra-chromosomal signals of *ABL1* [263]. Using RACE PCR, they identified in-frame fusions between the 5′ of *NUP214*, to 3′ *ABL1* (breakpoints in intron 1, similar to classic Ph+ ALL). The NUP214-ABL1 fusion protein is localised to the cytoplasmic side of the nuclear pore complex, has high ABL1 kinase activity, and is able to transform Ba/F3 cells, albeit with long latency [263, 264]. To date, 63 cases of T-ALL with *NUP214-ABL1* have been reported, with a frequency of approximately 5 % [265]. These cases are commonly associated with the *TLX1* and *TLX3* T-ALL subtypes, and affect paediatric and adult patients equally [265]. Because of their rarity, the prognostic significance of *NUP214-ABL1* is controversial in T-ALL, although two studies have suggested that poor prognosis is a feature particular to '*NUP214-ABL1* with hsr' (homogeneously staining regions, where the episomes have stably reintegrated into the genome). Interestingly, the only four T-ALL cell lines identified to harbour this fusion (ALL-SIL, PEER, Be-13 and TALL-104) all have '*NUP214-ABL1* with hsr', and all were established from cases of relapsed/refractory T-ALL. *NUP214-ABL1* positive T-ALL cell lines and Ba/F3 cells transformed by *NUP214-ABL1* undergo apoptosis in response to TKI treatment [263]. Case reports of clinical responses to imatinib and dasatinib in NUP214-ABL1 positive T-ALL suggest a clinical trial of TKIs is warranted in this patient subgroup [266, 267].

10.7 Future Directions

For over 30 years, chromosomal abnormalities have provided prognostic information in ALL, and this has been further refined over recent years, enabling a risk-adapted approach to treatment based on cytogenetic data [7]. Exactly how the spectrum of somatic mutations identified in both B- and T-ALL impacts on outcome is an area of ongoing study, but when combined with cytogenetic data and minimal residual disease analysis, should enable clinicians to stratify therapy more precisely. Furthermore, many of the newly discovered genomic lesions activate kinases, examples being NUP214-ABL1 in T-ALL and other ABL1/2 translocations in

Ph-like B-ALL, targetable by TKIs such as dasatanib; *FLT3* mutations in KMT2A-rearranged and hyperdiploid ALL, targetable by inhibitors such as sorafenib and quisartinib; and *CRLF2*/*IL7R* mutations/translocations targetable by JAK inhibitors such as ruxolitinib, suggesting that these agents warrant accelerated testing in clinical trials [252, 256, 259, 260, 268–271]. An alternative approach is to target chromatin modifying enzymes. For instance, overexpression of the *HMGN1* gene on trisomy 21, and in the high risk iAMP21, leads to depletion of H3K27me3, and sensitises cells to H3K27 demethylase inhibitors, an approach that also warrants testing in T-ALLs with loss-of-function mutations in *EZH2* [52].

However, many of the genes involved in translocations are transcription factors that have proven extremely challenging to target. Here, there has been an increased focus on identifying and targeting 'synthetic lethal' genes that are required specifically for cells expressing a particular oncogene. For instance, the DOT1L inhibitor, EPZ004777, a competitive inhibitor of the methyl donor S-adenosyl-methionine, has shown promise in pre-clinical studies of *KMT2A*-transformed ALL [272]. Another potentially druggable epigenetic target is KDM1A (LSD1), a lysine-specific demethylase involved in regulating KMT2A-transformed stem cells [272]. Pharmacological inhibition of LSD1 was shown to differentiate *KMT2A-AF9* cells, and clinical trials of ORY-1001 are in progress for relapsed AML [272]. Very recently, CDK6, a transcriptional target of KMT2A required for cell cycle progression, has been identified as a potential therapeutic target for KMT2A-related leukaemias, which has the potential to be rapidly tested in clinical trials, given that CDK6 inhibitors are already under clinical investigation in other cancers [274].

One potential hurdle of targeted therapy in ALL is that it is a clonally heterogeneous disease, meaning agents targeting the bulk of the leukaemia cell population, may select for outgrowth of subclones with self-renewal potential that do not harbour the targeted lesion. Thus, agents that target the initiating lesion (likely to be present in all clonally evolved cells), or that inhibit the leukaemic stem cell, may prove the most effective in yielding cures in very high risk ALL patients.

References

1. Armstrong SA, Look AT (2005) Molecular genetics of acute lymphoblastic leukemia. J Clin Oncol Off J Am Soc Clin Oncol 23(26):6306–6315
2. Ferrando AA, Look AT (2003) Gene expression profiling in T-cell acute lymphoblastic leukemia. Semin Hematol 40(4):274–280
3. Ferrando AA, Neuberg DS, Staunton J, Loh ML, Huard C, Raimondi SC et al (2002) Gene expression signatures define novel oncogenic pathways in T cell acute lymphoblastic leukemia. Cancer Cell 1(1):75–87
4. Zhang J, Ding L, Holmfeldt L, Wu G, Heatley SL, Payne-Turner D et al (2012) The genetic basis of early T-cell precursor acute lymphoblastic leukaemia. Nature 481(7380):157–163. Epub 2012/01/13
5. Meijerink JP (2010) Genetic rearrangements in relation to immunophenotype and outcome in T-cell acute lymphoblastic leukaemia. Best Pract Res Clin Haematol 23(3):307–318

6. Mullighan CG, Goorha S, Radtke I, Miller CB, Coustan-Smith E, Dalton JD et al (2007) Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. Nature 446(7137):758–764
7. Moorman AV, Ensor HM, Richards SM, Chilton L, Schwab C, Kinsey SE et al (2010) Prognostic effect of chromosomal abnormalities in childhood B-cell precursor acute lymphoblastic leukaemia: results from the UK Medical Research Council ALL97/99 randomised trial. Lancet Oncol 11(5):429–438. Epub 2010/04/23
8. Moricke A, Zimmermann M, Reiter A, Henze G, Schrauder A, Gadner H et al (2010) Long-term results of five consecutive trials in childhood acute lymphoblastic leukemia performed by the ALL-BFM study group from 1981 to 2000. Leuk Off J Leuk Soc Am Leuk Res Fund UK 24(2):265–284. Epub 2009/12/17
9. Goldstone AH, Richards SM, Lazarus HM, Tallman MS, Buck G, Fielding AK et al (2008) In adults with standard-risk acute lymphoblastic leukemia, the greatest benefit is achieved from a matched sibling allogeneic transplantation in first complete remission, and an autologous transplantation is less effective than conventional consolidation/maintenance chemotherapy in all patients: final results of the International ALL Trial (MRC UKALL XII/ECOG E2993). Blood 111(4):1827–1833. Epub 2007/12/01
10. Moorman AV (2012) The clinical relevance of chromosomal and genomic abnormalities in B-cell precursor acute lymphoblastic leukaemia. Blood Rev 26(3):123–135
11. Larmonie NS, Dik WA, Meijerink JP, Homminga I, van Dongen JJ, Langerak AW (2013) Breakpoint sites disclose the role of the V(D)J recombination machinery in the formation of T-cell receptor (TCR) and non-TCR associated aberrations in T-cell acute lymphoblastic leukemia. Haematologica 98(8):1173–1184. Epub 2013/08/02
12. Mansour MR, Sanda T, Lawton LN, Li X, Kreslavsky T, Novina CD et al (2013) The TAL1 complex targets the FBXW7 tumor suppressor by activating miR-223 in human T cell acute lymphoblastic leukemia. J Exp Med 210(8):1545–1557. Epub 2013/07/17
13. Herblot S, Steff AM, Hugo P, Aplan PD, Hoang T (2000) SCL and LMO1 alter thymocyte differentiation: inhibition of E2A-HEB function and pre-T alpha chain expression. Nat Immunol 1(2):138–144. Epub 2001/03/15
14. Porcher C, Swat W, Rockwell K, Fujiwara Y, Alt FW, Orkin SH (1996) The T cell leukemia oncoprotein SCL/tal-1 is essential for development of all hematopoietic lineages. Cell 86(1):47–57
15. Lacombe J, Herblot S, Rojas-Sutterlin S, Haman A, Barakat S, Iscove NN et al (2010) Scl regulates the quiescence and the long-term competence of hematopoietic stem cells. Blood 115(4):792–803. Epub 2009/10/24
16. Schatz DG (2004) V(D)J recombination. Immunol Rev 200:5–11
17. Schatz DG, Baltimore D (2004) Uncovering the V(D)J recombinase. Cell 116(2 Suppl):S103–S106, 2 p following S6
18. Akira S, Okazaki K, Sakano H (1987) Two pairs of recombination signals are sufficient to cause immunoglobulin V-(D)-J joining. Science 238(4830):1134–1138
19. Akamatsu Y, Tsurushita N, Nagawa F, Matsuoka M, Okazaki K, Imai M et al (1994) Essential residues in V(D)J recombination signals. J Immunol 153(10):4520–4529
20. Zhang Y, McCord RP, Ho YJ, Lajoie BR, Hildebrand DG, Simon AC et al (2012) Spatial organization of the mouse genome and its role in recurrent chromosomal translocations. Cell 148(5):908–921. Epub 2012/02/22
21. Alt FW, Zhang Y, Meng FL, Guo C, Schwer B (2013) Mechanisms of programmed DNA lesions and genomic instability in the immune system. Cell 152(3):417–429. Epub 2013/02/05
22. Marculescu R, Vanura K, Montpellier B, Roulland S, Le T, Navarro JM et al (2006) Recombinase, chromosomal translocations and lymphoid neoplasia: targeting mistakes and repair failures. DNA Repair 5(9–10):1246–1258. Epub 2006/06/27
23. Larmonie NS, van der Spek A, Bogers AJ, van Dongen JJ, Langerak AW (2014) Genetic and epigenetic determinants mediate proneness of oncogene breakpoint sites for involvement in TCR translocations. Genes Immun 15(2):72–81. Epub 2013/12/07

24. Jackson SP (2002) Sensing and repairing DNA double-strand breaks. Carcinogenesis 23(5):687–696. Epub 2002/05/23
25. Papaemmanuil E, Rapado I, Li Y, Potter NE, Wedge DC, Tubio J et al (2014) RAG-mediated recombination is the predominant driver of oncogenic rearrangement in ETV6-RUNX1 acute lymphoblastic leukemia. Nat Genet 46(2):116–125. Epub 2014/01/15
26. Vanura K, Montpellier B, Le T, Spicuglia S, Navarro JM, Cabaud O et al (2007) In vivo reinsertion of excised episomes by the V(D)J recombinase: a potential threat to genomic stability. PLoS Biol 5(3):e43. Epub 2007/02/15
27. Gale KB, Ford AM, Repp R, Borkhardt A, Keller C, Eden OB et al (1997) Backtracking leukemia to birth: identification of clonotypic gene fusion sequences in neonatal blood spots. Proc Natl Acad Sci U S A 94(25):13950–13954
28. Hong D, Gupta R, Ancliff P, Atzberger A, Brown J, Soneji S et al (2008) Initiating and cancer-propagating cells in TEL-AML1-associated childhood leukemia. Science 319(5861):336–339
29. Anderson K, Lutz C, van Delft FW, Bateman CM, Guo Y, Colman SM et al (2011) Genetic variegation of clonal architecture and propagating cells in leukaemia. Nature 469(7330):356–361. Epub 2010/12/17
30. Mansour MR, Duke V, Foroni L, Patel B, Allen CG, Ancliff PJ et al (2007) Notch-1 mutations are secondary events in some patients with T-cell acute lymphoblastic leukemia. Clin Cancer Res 13(23):6964–6969
31. Howe SJ, Mansour MR, Schwarzwaelder K, Bartholomae C, Hubank M, Kempski H et al (2008) Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. J Clin Invest 118(9):3143–3150
32. Mullighan CG, Phillips LA, Su X, Ma J, Miller CB, Shurtleff SA et al (2008) Genomic analysis of the clonal origins of relapsed acute lymphoblastic leukemia. Science 322(5906):1377–1380. Epub 2008/11/29
33. Eguchi-Ishimae M, Eguchi M, Kempski H, Greaves M (2008) NOTCH1 mutation can be an early, prenatal genetic event in T-ALL. Blood 111(1):376–378. Epub 2007/09/29
34. Trujillo JM, Ahearn MJ, Cork A (1974) General implications of chromosomal alterations in human leukemia. Hum Pathol 5(6):675–686. Epub 1974/11/01
35. Whang-Peng J, Knutsen T, Ziegler J, Leventhal B (1976) Cytogenetic studies in acute lymphocytic leukemia: special emphasis in long-term survival. Med Pediatr Oncol 2(3):333–351. Epub 1976/01/01
36. Look AT, Roberson PK, Williams DL, Rivera G, Bowman WP, Pui CH et al (1985) Prognostic importance of blast cell DNA content in childhood acute lymphoblastic leukemia. Blood 65(5):1079–1086. Epub 1985/05/01
37. Secker-Walker LM, Lawler SD, Hardisty RM (1978) Prognostic implications of chromosomal findings in acute lymphoblastic leukaemia at diagnosis. Br Med J 2(6151):1529–1530. Epub 1978/12/02
38. Sutcliffe MJ, Shuster JJ, Sather HN, Camitta BM, Pullen J, Schultz KR et al (2005) High concordance from independent studies by the Children's Cancer Group (CCG) and Pediatric Oncology Group (POG) associating favorable prognosis with combined trisomies 4, 10, and 17 in children with NCI standard-risk B-precursor acute lymphoblastic leukemia: a Children's Oncology Group (COG) initiative. Leukemia 19(5):734–740
39. Heerema NA, Nachman JB, Sather HN, Sensel MG, Lee MK, Hutchinson R et al (1999) Hypodiploidy with less than 45 chromosomes confers adverse risk in childhood acute lymphoblastic leukemia: a report from the children's cancer group. Blood 94(12):4036–4045
40. Ferrando AA, Look AT (2000) Clinical implications of recurring chromosomal and associated molecular abnormalities in acute lymphoblastic leukemia. Semin Hematol 37(4):381–395
41. Schultz KR, Pullen DJ, Sather HN, Shuster JJ, Devidas M, Borowitz MJ et al (2007) Risk- and response-based classification of childhood B-precursor acute lymphoblastic leukemia: a combined analysis of prognostic markers from the Pediatric Oncology Group (POG) and Children's Cancer Group (CCG). Blood 109(3):926–935

42. Pui CH, Carroll AJ, Head D, Raimondi SC, Shuster JJ, Crist WM et al (1990) Near-triploid and near-tetraploid acute lymphoblastic leukemia of childhood. Blood 76(3):590–596. Epub 1990/08/01
43. Trueworthy R, Shuster J, Look T, Crist W, Borowitz M, Carroll A et al (1992) Ploidy of lymphoblasts is the strongest predictor of treatment outcome in B-progenitor cell acute lymphoblastic leukemia of childhood: a Pediatric Oncology Group study. J Clin Oncol Off J Am Soc Clin Oncol 10(4):606–613
44. Raimondi SC, Pui CH, Hancock ML, Behm FG, Filatov L, Rivera GK (1996) Heterogeneity of hyperdiploid (51–67) childhood acute lymphoblastic leukemia. Leukemia 10(2):213–224
45. Martinez-Climent JA (1997) Molecular cytogenetics of childhood hematological malignancies. Leukemia 11(12):1999–2021
46. Armstrong SA, Mabon ME, Silverman LB, Li A, Gribben JG, Fox EA et al (2004) FLT3 mutations in childhood acute lymphoblastic leukemia. Blood 103(9):3544–3546
47. Taketani T, Taki T, Sugita K, Furuichi Y, Ishii E, Hanada R et al (2004) FLT3 mutations in the activation loop of tyrosine kinase domain are frequently found in infant ALL with MLL rearrangements and pediatric ALL with hyperdiploidy. Blood 103(3):1085–1088
48. Harrison CJ, Moorman AV, Broadfield ZJ, Cheung KL, Harris RL, Reza Jalali G et al (2004) Three distinct subgroups of hypodiploidy in acute lymphoblastic leukaemia. Br J Haematol 125(5):552–559. Epub 2004/05/19
49. Holmfeldt L, Wei L, Diaz-Flores E, Walsh M, Zhang J, Ding L et al (2013) The genomic landscape of hypodiploid acute lymphoblastic leukemia. Nat Genet 45(3):242–252. Epub 2013/01/22
50. Muhlbacher V, Zenger M, Schnittger S, Weissmann S, Kunze F, Kohlmann A et al (2014) Acute lymphoblastic leukemia with low hypodiploid/near triploid karyotype is a specific clinical entity and exhibits a very high TP53 mutation frequency of 93 %. Gene Chromosom Cancer 53(6):524–536. Epub 2014/03/13
51. Hertzberg L, Vendramini E, Ganmore I, Cazzaniga G, Schmitz M, Chalker J et al (2010) Down syndrome acute lymphoblastic leukemia, a highly heterogeneous disease in which aberrant expression of CRLF2 is associated with mutated JAK2: a report from the International BFM Study Group. Blood 115(5):1006–1017. Epub 2009/12/08
52. Lane AA, Chapuy B, Lin CY, Tivey T, Li H, Townsend EC et al (2014) Triplication of a 21q22 region contributes to B cell transformation through HMGN1 overexpression and loss of histone H3 Lys27 trimethylation. Nat Genet 46(6):618–623
53. Taub R, Kirsch I, Morton C, Lenoir G, Swan D, Tronick S et al (1982) Translocation of the c-myc gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells. Proc Natl Acad Sci U S A 79(24):7837–7841
54. Dalla-Favera R, Bregni M, Erikson J, Patterson D, Gallo RC, Croce CM (1982) Human c-myc onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. Proc Natl Acad Sci U S A 79(24):7824–7827
55. Mansour MR, Linch DC, Foroni L, Goldstone AH, Gale RE (2006) High incidence of Notch-1 mutations in adult patients with T-cell acute lymphoblastic leukemia. Leukemia 20(3):537–539
56. Weng AP, Ferrando AA, Lee W, Morris JP, Silverman LB, Sanchez-Irizarry C et al (2004) Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science 306(5694):269–271
57. Sharma VM, Calvo JA, Draheim KM, Cunningham LA, Hermance N, Beverly L et al (2006) Notch1 contributes to mouse T-cell leukemia by directly inducing the expression of c-myc. Mol Cell Biol 26(21):8022–8031
58. Palomero T, Lim WK, Odom DT, Sulis ML, Real PJ, Margolin A et al (2006) NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. Proc Natl Acad Sci U S A 103(48):18261–18266
59. Weng AP, Millholland JM, Yashiro-Ohtani Y, Arcangeli ML, Lau A, Wai C et al (2006) c-Myc is an important direct target of Notch1 in T-cell acute lymphoblastic leukemia/lymphoma. Genes Dev 20(15):2096–2109

60. Thompson BJ, Buonamici S, Sulis ML, Palomero T, Vilimas T, Basso G et al (2007) The SCFFBW7 ubiquitin ligase complex as a tumor suppressor in T cell leukemia. J Exp Med 204(8):1825–1835. Epub 2007/07/25
61. O'Neil J, Grim J, Strack P, Rao S, Tibbitts D, Winter C et al (2007) *FBW7* mutations in leukemic cells mediate NOTCH pathway activation and resistance to gamma-secretase inhibitors (in press)
62. Yada M, Hatakeyama S, Kamura T, Nishiyama M, Tsunematsu R, Imaki H et al (2004) Phosphorylation-dependent degradation of c-Myc is mediated by the F-box protein Fbw7. EMBO J 23(10):2116–2125. Epub 2004/04/23
63. Welcker M, Clurman BE (2008) FBW7 ubiquitin ligase: a tumour suppressor at the crossroads of cell division, growth and differentiation. Nat Rev Cancer 8(2):83–93
64. Nie Z, Hu G, Wei G, Cui K, Yamane A, Resch W et al (2012) c-Myc is a universal amplifier of expressed genes in lymphocytes and embryonic stem cells. Cell 151(1):68–79
65. Lin CY, Loven J, Rahl PB, Paranal RM, Burge CB, Bradner JE et al (2012) Transcriptional amplification in tumor cells with elevated c-Myc. Cell 151(1):56–67
66. Rahl PB, Lin CY, Seila AC, Flynn RA, McCuine S, Burge CB et al (2010) c-Myc regulates transcriptional pause release. Cell 141(3):432–445
67. Zhou Q, Li T, Price DH (2012) RNA polymerase II elongation control. Annu Rev Biochem 81:119–143
68. Russell LJ, Enshaei A, Jones L, Erhorn A, Masic D, Bentley H et al (2014) IGH@ translocations are prevalent in teenagers and young adults with acute lymphoblastic leukemia and are associated with a poor outcome. J Clin Oncol Off J Am Soc Clin Oncol 32:1453–1462
69. Szczepanski T, Pongers-Willemse MJ, Langerak AW, Harts WA, Wijkhuijs AJ, van Wering ER et al (1999) Ig heavy chain gene rearrangements in T-cell acute lymphoblastic leukemia exhibit predominant DH6-19 and DH7-27 gene usage, can result in complete V-D-J rearrangements, and are rare in T-cell receptor alpha beta lineage. Blood 93(12):4079–4085
70. Dyer MJ, Akasaka T, Capasso M, Dusanjh P, Lee YF, Karran EL et al (2010) Immunoglobulin heavy chain locus chromosomal translocations in B-cell precursor acute lymphoblastic leukemia: rare clinical curios or potent genetic drivers? Blood 115(8):1490–1499
71. Chen SS, Claus R, Lucas DM, Yu L, Qian J, Ruppert AS et al (2011) Silencing of the inhibitor of DNA binding protein 4 (ID4) contributes to the pathogenesis of mouse and human CLL. Blood 117(3):862–871
72. Lecuyer E, Hoang T (2004) SCL: from the origin of hematopoiesis to stem cells and leukemia. Exp Hematol 32(1):11–24. Epub 2004/01/17
73. Brown L, Cheng JT, Chen Q, Siciliano MJ, Crist W, Buchanan G et al (1990) Site-specific recombination of the tal-1 gene is a common occurrence in human T cell leukemia. EMBO J 9(10):3343–3351
74. Aplan PD, Lombardi DP, Kirsch IR (1991) Structural characterization of SIL, a gene frequently disrupted in T-cell acute lymphoblastic leukemia. Mol Cell Biol 11(11):5462–5469
75. Breit TM, Beishuizen A, Ludwig WD, Mol EJ, Adriaansen HJ, van Wering ER et al (1993) tal-1 deletions in T-cell acute lymphoblastic leukemia as PCR target for detection of minimal residual disease. Leuk Off J Leuk Soc Am Leuk Res Fund UK 7(12):2004–2011. Epub 1993/12/01
76. Breit TM, Mol EJ, Wolvers-Tettero IL, Ludwig WD, van Wering ER, van Dongen JJ (1993) Site-specific deletions involving the tal-1 and sil genes are restricted to cells of the T cell receptor alpha/beta lineage: T cell receptor delta gene deletion mechanism affects multiple genes. J Exp Med 177(4):965–977
77. Aifantis I, Raetz E, Buonamici S (2008) Molecular pathogenesis of T-cell leukaemia and lymphoma. Nat Rev Immunol 8(5):380–390. Epub 2008/04/19
78. Mansour MR, Abraham BJ, Anders L, Berezovskaya A, Gutierrez A, Durbin AD, Etchin J, Lawton L, Sallan SE, Silverman LB, Loh ML, Hunger SP, Sanda S, Young RA, Look AT (2014) An oncogenic super-enhancer formed through somatic mutation of a noncoding intergenic element. Science 346(6215):1373–1377

79. Hsu HL, Cheng JT, Chen Q, Baer R (1991) Enhancer-binding activity of the tal-1 oncoprotein in association with the E47/E12 helix-loop-helix proteins. Mol Cell Biol 11(6):3037–3042. Epub 1991/06/01
80. Hsu HL, Wadman I, Baer R (1994) Formation of in vivo complexes between the TAL1 and E2A polypeptides of leukemic T cells. Proc Natl Acad Sci U S A 91(8):3181–3185. Epub 1994/04/12
81. Huang S, Brandt SJ (2000) mSin3A regulates murine erythroleukemia cell differentiation through association with the TAL1 (or SCL) transcription factor. Mol Cell Biol 20(6):2248–2259. Epub 2000/02/25
82. Huang S, Qiu Y, Stein RW, Brandt SJ (1999) p300 functions as a transcriptional coactivator for the TAL1/SCL oncoprotein. Oncogene 18(35):4958–4967. Epub 1999/09/22
83. Sanda T, Lawton LN, Barrasa MI, Fan ZP, Kohlhammer H, Gutierrez A et al (2012) Core transcriptional regulatory circuit controlled by the TAL1 complex in human T cell acute lymphoblastic leukemia. Cancer Cell 22(2):209–221. Epub 2012/08/18
84. Kusy S, Gerby B, Goardon N, Gault N, Ferri F, Gerard D et al (2010) NKX3.1 is a direct TAL1 target gene that mediates proliferation of TAL1-expressing human T cell acute lymphoblastic leukemia. J Exp Med 207(10):2141–2156. Epub 2010/09/22
85. Mavrakis KJ, Van Der Meulen J, Wolfe AL, Liu X, Mets E, Taghon T et al (2011) A cooperative microRNA-tumor suppressor gene network in acute T-cell lymphoblastic leukemia (T-ALL). Nat Genet 43(7):673–678. Epub 2011/06/07
86. Nam CH, Rabbitts TH (2006) The role of LMO2 in development and in T cell leukemia after chromosomal translocation or retroviral insertion. Mol Ther J Am Soc Gene Ther 13(1):15–25. Epub 2005/11/02
87. McCormack MP, Forster A, Drynan L, Pannell R, Rabbitts TH (2003) The LMO2 T-cell oncogene is activated via chromosomal translocations or retroviral insertion during gene therapy but has no mandatory role in normal T-cell development. Mol Cell Biol 23(24):9003–9013. Epub 2003/12/04
88. Rabbitts TH (1998) LMO T-cell translocation oncogenes typify genes activated by chromosomal translocations that alter transcription and developmental processes. Genes Dev 12(17):2651–2657. Epub 1998/09/10
89. Hammond SM, Crable SC, Anderson KP (2005) Negative regulatory elements are present in the human LMO2 oncogene and may contribute to its expression in leukemia. Leuk Res 29(1):89–97
90. Dik WA, Nadel B, Przybylski GK, Asnafi V, Grabarczyk P, Navarro JM et al (2007) Different chromosomal breakpoints impact the level of LMO2 expression in T-ALL. Blood 110(1):388–392
91. Van Vlierberghe P, van Grotel M, Beverloo HB, Lee C, Helgason T, Buijs-Gladdines J et al (2006) The cryptic chromosomal deletion del(11)(p12p13) as a new activation mechanism of LMO2 in pediatric T-cell acute lymphoblastic leukemia. Blood 108(10):3520–3529
92. Hacein-Bey-Abina S, de Saint BG, Cavazzana-Calvo M (2003) Gene therapy of X-linked severe combined immunodeficiency. Methods Mol Biol 215:247–259. Epub 2003/01/07
93. Hacein-Bey-Abina S, Garrigue A, Wang GP, Soulier J, Lim A, Morillon E et al (2008) Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. J Clin Invest 118(9):3132–3142. Epub 2008/08/09
94. Hacein-Bey-Abina S, von Kalle C, Schmidt M, Le Deist F, Wulffraat N, McIntyre E et al (2003) A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. N Engl J Med 348(3):255–256. Epub 2003/01/17
95. Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P et al (2003) LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science 302(5644):415–419. Epub 2003/10/18
96. Wadman I, Li J, Bash RO, Forster A, Osada H, Rabbitts TH et al (1994) Specific in vivo association between the bHLH and LIM proteins implicated in human T cell leukemia. EMBO J 13(20):4831–4839

97. Valge-Archer VE, Osada H, Warren AJ, Forster A, Li J, Baer R et al (1994) The LIM protein RBTN2 and the basic helix-loop-helix protein TAL1 are present in a complex in erythroid cells. Proc Natl Acad Sci U S A 91(18):8617–8621
98. Larson RC, Lavenir I, Larson TA, Baer R, Warren AJ, Wadman I et al (1996) Protein dimerization between Lmo2 (Rbtn2) and Tal1 alters thymocyte development and potentiates T cell tumorigenesis in transgenic mice. EMBO J 15(5):1021–1027
99. Homminga I, Pieters R, Langerak AW, de Rooi JJ, Stubbs A, Verstegen M et al (2011) Integrated transcript and genome analyses reveal NKX2-1 and MEF2C as potential oncogenes in T cell acute lymphoblastic leukemia. Cancer Cell 19(4):484–497. Epub 2011/04/13
100. McGuire EA, Rintoul CE, Sclar GM, Korsmeyer SJ (1992) Thymic overexpression of Ttg-1 in transgenic mice results in T-cell acute lymphoblastic leukemia/lymphoma. Mol Cell Biol 12(9):4186–4196
101. Fisch P, Boehm T, Lavenir I, Larson T, Arno J, Forster A et al (1992) T-cell acute lymphoblastic lymphoma induced in transgenic mice by the RBTN1 and RBTN2 LIM-domain genes. Oncogene 7(12):2389–2397
102. Larson RC, Fisch P, Larson TA, Lavenir I, Langford T, King G et al (1994) T cell tumours of disparate phenotype in mice transgenic for Rbtn-2. Oncogene 9(12):3675–3681
103. Larson RC, Osada H, Larson TA, Lavenir I, Rabbitts TH (1995) The oncogenic LIM protein Rbtn2 causes thymic developmental aberrations that precede malignancy in transgenic mice. Oncogene 11(5):853–862
104. Neale GA, Rehg JE, Goorha RM (1995) Ectopic expression of rhombotin-2 causes selective expansion of CD4-CD8- lymphocytes in the thymus and T-cell tumors in transgenic mice. Blood 86(8):3060–3071
105. McCormack MP, Young LF, Vasudevan S, de Graaf CA, Codrington R, Rabbitts TH et al (2010) The Lmo2 oncogene initiates leukemia in mice by inducing thymocyte self-renewal. Science 327(5967):879–883
106. Smith S, Tripathi R, Goodings C, Cleveland S, Mathias E, Hardaway JA et al (2014) LIM domain only-2 (LMO2) induces T-cell leukemia by two distinct pathways. PLoS One 9(1):e85883
107. O'Neil J, Calvo J, McKenna K, Krishnamoorthy V, Aster JC, Bassing CH et al (2006) Activating Notch1 mutations in mouse models of T-ALL. Blood 107(2):781–785. Epub 2005/09/17
108. Ferrando AA, Neuberg DS, Dodge RK, Paietta E, Larson RA, Wiernik PH et al (2004) Prognostic importance of TLX1 (HOX11) oncogene expression in adults with T-cell acute lymphoblastic leukaemia. Lancet 363(9408):535–536. Epub 2004/02/21
109. Ferrando AA, Herblot S, Palomero T, Hansen M, Hoang T, Fox EA et al (2004) Biallelic transcriptional activation of oncogenic transcription factors in T-cell acute lymphoblastic leukemia. Blood 103(5):1909–1911
110. Kees UR, Heerema NA, Kumar R, Watt PM, Baker DL, La MK et al (2003) Expression of HOX11 in childhood T-lineage acute lymphoblastic leukaemia can occur in the absence of cytogenetic aberration at 10q24: a study from the Children's Cancer Group (CCG). Leukemia 17(5):887–893
111. Asnafi V, Buzyn A, Le Noir S, Baleydier F, Simon A, Beldjord K et al (2009) NOTCH1/FBXW7 mutation identifies a large subgroup with favorable outcome in adult T-cell acute lymphoblastic leukemia (T-ALL): a Group for Research on Adult Acute Lymphoblastic Leukemia (GRAALL) study. Blood 113(17):3918–3924. Epub 2008/12/26
112. De Keersmaecker K, Real PJ, Gatta GD, Palomero T, Sulis ML, Tosello V et al (2010) The TLX1 oncogene drives aneuploidy in T cell transformation. Nat Med 16(11):1321–1327. Epub 2010/10/26
113. Berger R, Dastugue N, Busson M, Van Den Akker J, Perot C, Ballerini P et al (2003) t(5;14)/HOX11L2-positive T-cell acute lymphoblastic leukemia. A collaborative study of the Groupe Francais de Cytogenetique Hematologique (GFCH). Leukemia 17(9):1851–1857
114. Ballerini P, Blaise A, Busson-Le Coniat M, Su XY, Zucman-Rossi J, Adam M et al (2002) HOX11L2 expression defines a clinical subtype of pediatric T-ALL associated with poor prognosis. Blood 100(3):991–997

115. Mauvieux L, Leymarie V, Helias C, Perrusson N, Falkenrodt A, Lioure B et al (2002) High incidence of Hox11L2 expression in children with T-ALL. Leukemia 16(12):2417–2422
116. Cave H, Suciu S, Preudhomme C, Poppe B, Robert A, Uyttebroeck A et al (2004) Clinical significance of HOX11L2 expression linked to t(5;14)(q35;q32), of HOX11 expression, and of SIL-TAL fusion in childhood T-cell malignancies: results of EORTC studies 58881 and 58951. Blood 103(2):442–450
117. Su XY, Della-Valle V, Andre-Schmutz I, Lemercier C, Radford-Weiss I, Ballerini P et al (2006) HOX11L2/TLX3 is transcriptionally activated through T-cell regulatory elements downstream of BCL11B as a result of the t(5;14)(q35;q32). Blood 108(13):4198–4201. Epub 2006/08/24
118. Bernard OA, Busson-LeConiat M, Ballerini P, Mauchauffe M, Della Valle V, Monni R et al (2001) A new recurrent and specific cryptic translocation, t(5;14)(q35;q32), is associated with expression of the Hox11L2 gene in T acute lymphoblastic leukemia. Leukemia 15(10):1495–1504
119. Su XY, Busson M, Della Valle V, Ballerini P, Dastugue N, Talmant P et al (2004) Various types of rearrangements target TLX3 locus in T-cell acute lymphoblastic leukemia. Genes Chromosomes Cancer 41(3):243–249
120. Attarbaschi A, Pisecker M, Inthal A, Mann G, Janousek D, Dworzak M et al (2010) Prognostic relevance of TLX3 (HOX11L2) expression in childhood T-cell acute lymphoblastic leukaemia treated with Berlin-Frankfurt-Munster (BFM) protocols containing early and late re-intensification elements. Br J Haematol 148(2):293–300
121. Gottardo NG, Jacoby PA, Sather HN, Reaman GH, Baker DL, Kees UR (2005) Significance of HOX11L2/TLX3 expression in children with T-cell acute lymphoblastic leukemia treated on Children's Cancer Group protocols. Leukemia 19(9):1705–1708
122. Shurtleff SA, Buijs A, Behm FG, Rubnitz JE, Raimondi SC, Hancock ML et al (1995) TEL/AML1 fusion resulting from a cryptic t(12;21) is the most common genetic lesion in pediatric ALL and defines a subgroup of patients with an excellent prognosis. Leukemia 9(12):1985–1989
123. Golub TR, Barker GF, Bohlander SK, Hiebert SW, Ward DC, Bray-Ward P et al (1995) Fusion of the TEL gene on 12p13 to the AML1 gene on 21q22 in acute lymphoblastic leukemia. Proc Natl Acad Sci U S A 92(11):4917–4921
124. Fears S, Vignon C, Bohlander SK, Smith S, Rowley JD, Nucifora G (1996) Correlation between the ETV6/CBFA2 (TEL/AML1) fusion gene and karyotypic abnormalities in children with B-cell precursor acute lymphoblastic leukemia. Genes Chromosomes Cancer 17(2):127–135
125. Romana SP, Poirel H, Leconiat M, Flexor MA, Mauchauffe M, Jonveaux P et al (1995) High frequency of t(12;21) in childhood B-lineage acute lymphoblastic leukemia. Blood 86(11):4263–4269
126. Cave H, Cacheux V, Raynaud S, Brunie G, Bakkus M, Cochaux P et al (1997) ETV6 is the target of chromosome 12p deletions in t(12;21) childhood acute lymphocytic leukemia. Leukemia 11(9):1459–1464
127. Jousset C, Carron C, Boureux A, Quang CT, Oury C, Dusanter-Fourt I et al (1997) A domain of TEL conserved in a subset of ETS proteins defines a specific oligomerization interface essential to the mitogenic properties of the TEL-PDGFR beta oncoprotein. EMBO J 16(1):69–82
128. McLean TW, Ringold S, Neuberg D, Stegmaier K, Tantravahi R, Ritz J et al (1996) TEL/AML-1 dimerizes and is associated with a favorable outcome in childhood acute lymphoblastic leukemia. Blood 88(11):4252–4258
129. Takeuchi S, Seriu T, Bartram CR, Golub TR, Reiter A, Miyoshi I et al (1997) TEL is one of the targets for deletion on 12p in many cases of childhood B-lineage acute lymphoblastic leukemia. Leukemia 11(8):1220–1223

130. Berger R, Bernheim A, Daniel MT, Valensi F, Sigaux F, Flandrin G (1982) Cytologic characterization and significance of normal karyotypes in t(8;21) acute myeloblastic leukemia. Blood 59(1):171–178
131. Nucifora G, Rowley JD (1995) AML1 and the 8;21 and 3;21 translocations in acute and chronic myeloid leukemia. Blood 86(1):1–14
132. Fisher AL, Caudy M (1998) Groucho proteins: transcriptional corepressors for specific subsets of DNA-binding transcription factors in vertebrates and invertebrates. Genes Dev 12(13):1931–1940
133. Zelent A, Greaves M, Enver T (2004) Role of the TEL-AML1 fusion gene in the molecular pathogenesis of childhood acute lymphoblastic leukaemia. Oncogene 23(24):4275–4283
134. Imai Y, Kurokawa M, Tanaka K, Friedman AD, Ogawa S, Mitani K et al (1998) TLE, the human homolog of groucho, interacts with AML1 and acts as a repressor of AML1-induced transactivation. Biochem Biophys Res Commun 252(3):582–589
135. Hiebert SW, Sun W, Davis JN, Golub T, Shurtleff S, Buijs A et al (1996) The t(12;21) translocation converts AML-1B from an activator to a repressor of transcription. Mol Cell Biol 16(4):1349–1355
136. Andreasson P, Schwaller J, Anastasiadou E, Aster J, Gilliland DG (2001) The expression of ETV6/CBFA2 (TEL/AML1) is not sufficient for the transformation of hematopoietic cell lines in vitro or the induction of hematologic disease in vivo. Cancer Genet Cytogenet 130(2):93–104
137. Ford AM, Palmi C, Bueno C, Hong D, Cardus P, Knight D et al (2009) The TEL-AML1 leukemia fusion gene dysregulates the TGF-beta pathway in early B lineage progenitor cells. J Clin Invest 119(4):826–836
138. Mori H, Colman SM, Xiao Z, Ford AM, Healy LE, Donaldson C et al (2002) Chromosome translocations and covert leukemic clones are generated during normal fetal development. Proc Natl Acad Sci U S A 99(12):8242–8247
139. Loh ML, Goldwasser MA, Silverman LB, Poon WM, Vattikuti S, Cardoso A et al (2006) Prospective analysis of TEL/AML1-positive patients treated on Dana-Farber Cancer Institute Consortium protocol 95-01. Blood 107(11):4508–4513
140. Asakura K, Uchida H, Miyachi H, Kobayashi H, Miyakawa Y, Nimer SD et al (2004) TEL/AML1 overcomes drug resistance through transcriptional repression of multidrug resistance-1 gene expression. Mol Cancer Res 2(6):339–347
141. Zaza G, Yang W, Kager L, Cheok M, Downing J, Pui CH et al (2004) Acute lymphoblastic leukemia with TEL-AML1 fusion has lower expression of genes involved in purine metabolism and lower de novo purine synthesis. Blood 104(5):1435–1441
142. Kamps MP, Look AT, Baltimore D (1991) The human t(1;19) translocation in pre-B ALL produces multiple nuclear E2A-Pbx1 fusion proteins with differing transforming potentials. Genes Dev 5(3):358–368
143. Raimondi SC, Behm FG, Roberson PK, Williams DL, Pui CH, Crist WM et al (1990) Cytogenetics of pre-B-cell acute lymphoblastic leukemia with emphasis on prognostic implications of the t(1;19). J Clin Oncol Off J Am Soc Clin Oncol 8(8):1380–1388
144. Uckun FM, Sensel MG, Sather HN, Gaynon PS, Arthur DC, Lange BJ et al (1998) Clinical significance of translocation t(1;19) in childhood acute lymphoblastic leukemia in the context of contemporary therapies: a report from the Children's Cancer Group. J Clin Oncol Off J Am Soc Clin Oncol 16(2):527–535
145. Moorman AV, Harrison CJ, Buck GA, Richards SM, Secker-Walker LM, Martineau M et al (2007) Karyotype is an independent prognostic factor in adult acute lymphoblastic leukemia (ALL): analysis of cytogenetic data from patients treated on the Medical Research Council (MRC) UKALLXII/Eastern Cooperative Oncology Group (ECOG) 2993 trial. Blood 109(8):3189–3197. Epub 2006/12/16
146. Crist WM, Carroll AJ, Shuster JJ, Behm FG, Whitehead M, Vietti TJ et al (1990) Poor prognosis of children with pre-B acute lymphoblastic leukemia is associated with the t(1;19)(q23;p13): a Pediatric Oncology Group study. Blood 76(1):117–122

147. Pui CH, Crist WM (1992) Cytogenetic abnormalities in childhood acute lymphoblastic leukemia correlates with clinical features and treatment outcome. Leuk Lymphoma 7(4):259–274
148. Rivera GK, Raimondi SC, Hancock ML, Behm FG, Pui CH, Abromowitch M et al (1991) Improved outcome in childhood acute lymphoblastic leukaemia with reinforced early treatment and rotational combination chemotherapy. Lancet 337(8733):61–66
149. Kamps MP, Murre C, Sun XH, Baltimore D (1990) A new homeobox gene contributes the DNA binding domain of the t(1;19) translocation protein in pre-B ALL. Cell 60(4):547–555
150. Nourse J, Mellentin JD, Galili N, Wilkinson J, Stanbridge E, Smith SD et al (1990) Chromosomal translocation t(1;19) results in synthesis of a homeobox fusion mRNA that codes for a potential chimeric transcription factor. Cell 60(4):535–545
151. Mellentin JD, Nourse J, Hunger SP, Smith SD, Cleary ML (1990) Molecular analysis of the t(1;19) breakpoint cluster region in pre-B cell acute lymphoblastic leukemias. Genes Chromosomes Cancer 2(3):239–247
152. Monica K, LeBrun DP, Dedera DA, Brown R, Cleary ML (1994) Transformation properties of the E2a-Pbx1 chimeric oncoprotein: fusion with E2a is essential, but the Pbx1 homeodomain is dispensable. Mol Cell Biol 14(12):8304–8314
153. Chang CP, de Vivo I, Cleary ML (1997) The Hox cooperativity motif of the chimeric oncoprotein E2a-Pbx1 is necessary and sufficient for oncogenesis. Mol Cell Biol 17(1):81–88
154. Kamps MP, Baltimore D (1993) E2A-Pbx1, the t(1;19) translocation protein of human pre-B-cell acute lymphocytic leukemia, causes acute myeloid leukemia in mice. Mol Cell Biol 13(1):351–357
155. Dedera DA, Waller EK, LeBrun DP, Sen-Majumdar A, Stevens ME, Barsh GS et al (1993) Chimeric homeobox gene E2A-PBX1 induces proliferation, apoptosis, and malignant lymphomas in transgenic mice. Cell 74(5):833–843
156. Smith KS, Jacobs Y, Chang CP, Cleary ML (1997) Chimeric oncoprotein E2a-Pbx1 induces apoptosis of hematopoietic cells by a p53-independent mechanism that is suppressed by Bcl-2. Oncogene 14(24):2917–2926. Epub 1997/06/19
157. Hunger SP (1996) Chromosomal translocations involving the E2A gene in acute lymphoblastic leukemia: clinical features and molecular pathogenesis. Blood 87(4):1211–1224
158. Inukai T, Hirose K, Inaba T, Kurosawa H, Hama A, Inada H et al (2007) Hypercalcemia in childhood acute lymphoblastic leukemia: frequent implication of parathyroid hormone-related peptide and E2A-HLF from translocation 17;19. Leukemia 21(2):288–296. Epub 2006/12/22
159. Akahane K, Inukai T, Inaba T, Kurosawa H, Look AT, Kiyokawa N et al (2010) Specific induction of CD33 expression by E2A-HLF: the first evidence for aberrant myeloid antigen expression in ALL by a fusion transcription factor. Leukemia 24(4):865–869. Epub 2010/02/12
160. Massari ME, Jennings PA, Murre C (1996) The AD1 transactivation domain of E2A contains a highly conserved helix which is required for its activity in both Saccharomyces cerevisiae and mammalian cells. Mol Cell Biol 16(1):121–129. Epub 1996/01/01
161. Aronheim A, Shiran R, Rosen A, Walker MD (1993) The E2A gene product contains two separable and functionally distinct transcription activation domains. Proc Natl Acad Sci U S A 90(17):8063–8067. Epub 1993/09/01
162. Inukai T, Inaba T, Yoshihara T, Look AT (1997) Cell transformation mediated by homodimeric E2A-HLF transcription factors. Mol Cell Biol 17(3):1417–1424. Epub 1997/03/01
163. Yoshihara T, Inaba T, Shapiro LH, Kato JY, Look AT (1995) E2A-HLF-mediated cell transformation requires both the trans-activation domains of E2A and the leucine zipper dimerization domain of HLF. Mol Cell Biol 15(6):3247–3255
164. Smith KS, Rhee JW, Cleary ML (2002) Transformation of bone marrow B-cell progenitors by E2a-Hlf requires coexpression of Bcl-2. Mol Cell Biol 22(21):7678–7687. Epub 2002/10/09

165. de Boer J, Yeung J, Ellu J, Ramanujachar R, Bornhauser B, Solarska O et al (2011) The E2A-HLF oncogenic fusion protein acts through Lmo2 and Bcl-2 to immortalize hematopoietic progenitors. Leukemia 25(2):321–330. Epub 2010/11/13
166. Okuya M, Kurosawa H, Kikuchi J, Furukawa Y, Matsui H, Aki D et al (2010) Up-regulation of survivin by the E2A-HLF chimera is indispensable for the survival of t(17;19)-positive leukemia cells. J Biol Chem 285(3):1850–1860. Epub 2009/11/06
167. Inukai T, Inoue A, Kurosawa H, Goi K, Shinjyo T, Ozawa K et al (1999) SLUG, a ces-1-related zinc finger transcription factor gene with antiapoptotic activity, is a downstream target of the E2A-HLF oncoprotein. Mol Cell 4(3):343–352
168. Inoue A, Seidel MG, Wu W, Kamizono S, Ferrando AA, Bronson RT et al (2002) Slug, a highly conserved zinc finger transcriptional repressor, protects hematopoietic progenitor cells from radiation-induced apoptosis in vivo. Cancer Cell 2(4):279–288
169. Souers AJ, Leverson JD, Boghaert ER, Ackler SL, Catron ND, Chen J et al (2013) ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets. Nat Med 19(2):202–208. Epub 2013/01/08
170. Ziemin-van der Poel S, McCabe NR, Gill HJ, Espinosa R III, Patel Y, Harden A et al (1991) Identification of a gene, MLL, that spans the breakpoint in 11q23 translocations associated with human leukemias. Proc Natl Acad Sci U S A 88(23):10735–10739
171. Tkachuk DC, Kohler S, Cleary ML (1992) Involvement of a homolog of Drosophila trithorax by 11q23 chromosomal translocations in acute leukemias. Cell 71(4):691–700
172. Gu Y, Nakamura T, Alder H, Prasad R, Canaani O, Cimino G et al (1992) The t(4;11) chromosome translocation of human acute leukemias fuses the ALL-1 gene, related to Drosophila trithorax, to the AF-4 gene. Cell 71(4):701–708
173. Djabali M, Selleri L, Parry P, Bower M, Young BD, Evans GA (1992) A trithorax-like gene is interrupted by chromosome 11q23 translocations in acute leukaemias. Nat Genet 2(2):113–118
174. Rowley JD, Reshmi S, Sobulo O, Musvee T, Anastasi J, Raimondi S et al (1997) All patients with the T(11;16)(q23;p13.3) that involves MLL and CBP have treatment-related hematologic disorders. Blood 90(2):535–541
175. Pedersen-Bjergaard J, Philip P (1991) Balanced translocations involving chromosome bands 11q23 and 21q22 are highly characteristic of myelodysplasia and leukemia following therapy with cytostatic agents targeting at DNA-topoisomerase II. Blood 78(4):1147–1148
176. Harper DP, Aplan PD (2008) Chromosomal rearrangements leading to MLL gene fusions: clinical and biological aspects. Cancer Res 68(24):10024–10027
177. Tomizawa D, Koh K, Sato T, Kinukawa N, Morimoto A, Isoyama K et al (2007) Outcome of risk-based therapy for infant acute lymphoblastic leukemia with or without an MLL gene rearrangement, with emphasis on late effects: a final report of two consecutive studies, MLL96 and MLL98, of the Japan Infant Leukemia Study Group. Leukemia 21(11):2258–2263
178. Greaves MF (1996) Infant leukaemia biology, aetiology and treatment. Leukemia 10(2):372–377
179. Greaves MF, Maia AT, Wiemels JL, Ford AM (2003) Leukemia in twins: lessons in natural history. Blood 102(7):2321–2333
180. Hsieh JJ, Ernst P, Erdjument-Bromage H, Tempst P, Korsmeyer SJ (2003) Proteolytic cleavage of MLL generates a complex of N- and C-terminal fragments that confers protein stability and subnuclear localization. Mol Cell Biol 23(1):186–194
181. Nakamura T, Mori T, Tada S, Krajewski W, Rozovskaia T, Wassell R et al (2002) ALL-1 is a histone methyltransferase that assembles a supercomplex of proteins involved in transcriptional regulation. Mol Cell 10(5):1119–1128. Epub 2002/11/28
182. Somervaille TC, Cleary ML (2010) Grist for the MLL: how do MLL oncogenic fusion proteins generate leukemia stem cells? Int J Hematol 91(5):735–741. Epub 2010/05/11
183. Johansson B, Moorman AV, Secker-Walker LM (1998) Derivative chromosomes of 11q23-translocations in hematologic malignancies. European 11q23 Workshop participants. Leukemia 12(5):828–833. PubMed PMID: 9593288

184. Marschalek R (2011) Mechanisms of leukemogenesis by MLL fusion proteins. Br J Haematol 152(2):141–154
185. Corral J, Lavenir I, Impey H, Warren AJ, Forster A, Larson TA et al (1996) An Mll-AF9 fusion gene made by homologous recombination causes acute leukemia in chimeric mice: a method to create fusion oncogenes. Cell 85(6):853–861
186. Zeisig BB, Garcia-Cuellar MP, Winkler TH, Slany RK (2003) The oncoprotein MLL-ENL disturbs hematopoietic lineage determination and transforms a biphenotypic lymphoid/myeloid cell. Oncogene 22(11):1629–1637
187. Lavau C, Luo RT, Du C, Thirman MJ (2000) Retrovirus-mediated gene transfer of MLL-ELL transforms primary myeloid progenitors and causes acute myeloid leukemias in mice. Proc Natl Acad Sci U S A 97(20):10984–10989
188. Lavau C, Du C, Thirman M, Zeleznik-Le N (2000) Chromatin-related properties of CBP fused to MLL generate a myelodysplastic-like syndrome that evolves into myeloid leukemia. EMBO J 19(17):4655–4664
189. Ayton PM, Cleary ML (2003) Transformation of myeloid progenitors by MLL oncoproteins is dependent on Hoxa7 and Hoxa9. Genes Dev 17(18):2298–2307
190. Kumar AR, Hudson WA, Chen W, Nishiuchi R, Yao Q, Kersey JH (2004) Hoxa9 influences the phenotype but not the incidence of Mll-AF9 fusion gene leukemia. Blood 103(5):1823–1828
191. Armstrong SA, Staunton JE, Silverman LB, Pieters R, den Boer ML, Minden MD et al (2002) MLL translocations specify a distinct gene expression profile that distinguishes a unique leukemia. Nat Genet 30(1):41–47
192. Yeoh EJ, Ross ME, Shurtleff SA, Williams WK, Patel D, Mahfouz R et al (2002) Classification, subtype discovery, and prediction of outcome in pediatric acute lymphoblastic leukemia by gene expression profiling. Cancer Cell 1(2):133–143
193. Ferrando AA, Armstrong SA, Neuberg DS, Sallan SE, Silverman LB, Korsmeyer SJ et al (2003) Gene expression signatures in MLL-rearranged T-lineage and B-precursor acute leukemias: dominance of HOX dysregulation. Blood 102(1):262–268
194. Bernt KM, Armstrong SA (2011) Targeting epigenetic programs in MLL-rearranged leukemias. Hematol Am Soc Hematol Educ Program 2011:354–360
195. Okada Y, Feng Q, Lin Y, Jiang Q, Li Y, Coffield VM et al (2005) hDOT1L links histone methylation to leukemogenesis. Cell 121(2):167–178. Epub 2005/04/27
196. Krivtsov AV, Feng Z, Lemieux ME, Faber J, Vempati S, Sinha AU et al (2008) H3K79 methylation profiles define murine and human MLL-AF4 leukemias. Cancer Cell 14(5):355–368. Epub 2008/11/04
197. Mann G, Attarbaschi A, Schrappe M, De Lorenzo P, Peters C, Hann I et al (2010) Improved outcome with hematopoietic stem cell transplantation in a poor prognostic subgroup of infants with mixed-lineage-leukemia (MLL)-rearranged acute lymphoblastic leukemia: results from the Interfant-99 Study. Blood 116(15):2644–2650
198. Pui CH, Gaynon PS, Boyett JM, Chessells JM, Baruchel A, Kamps W et al (2002) Outcome of treatment in childhood acute lymphoblastic leukaemia with rearrangements of the 11q23 chromosomal region. Lancet 359(9321):1909–1915
199. Pui CH (1991) Epipodophyllotoxin-related acute myeloid leukaemia. Lancet 338(8780):1468
200. Moorman AV, Hagemeijer A, Charrin C, Rieder H, Secker-Walker LM (1998) The translocations, t(11;19)(q23;p13.1) and t(11;19)(q23;p13.3): a cytogenetic and clinical profile of 53 patients. European 11q23 Workshop participants. Leukemia 12(5):805–810
201. Nowell PC, Hungerford DA (1961) Chromosome studies in human leukemia. II. Chronic granulocytic leukemia. J Natl Cancer Inst 27:1013–1035
202. Rowley JD (1973) Letter: a new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. Nature 243(5405):290–293
203. de Klein A, van Kessel AG, Grosveld G, Bartram CR, Hagemeijer A, Bootsma D et al (1982) A cellular oncogene is translocated to the Philadelphia chromosome in chronic myelocytic leukaemia. Nature 300(5894):765–767. Epub 1982/12/23

204. Burmeister T, Schwartz S, Bartram CR, Gokbuget N, Hoelzer D, Thiel E et al (2008) Patients' age and BCR-ABL frequency in adult B-precursor ALL: a retrospective analysis from the GMALL study group. Blood 112(3):918–919
205. Raanani P, Trakhtenbrot L, Rechavi G, Rosenthal E, Avigdor A, Brok-Simoni F et al (2005) Philadelphia-chromosome-positive T-lymphoblastic leukemia: acute leukemia or chronic myelogenous leukemia blastic crisis. Acta Haematol 113(3):181–189
206. Heisterkamp N, Stephenson JR, Groffen J, Hansen PF, de Klein A, Bartram CR et al (1983) Localization of the c-ab1 oncogene adjacent to a translocation break point in chronic myelocytic leukaemia. Nature 306(5940):239–242
207. Leibowitz D, Schaefer-Rego K, Popenoe DW, Mears JG, Bank A (1985) Variable breakpoints on the Philadelphia chromosome in chronic myelogenous leukemia. Blood 66(1):243–245
208. Grosveld G, Verwoerd T, van Agthoven T, de Klein A, Ramachandran KL, Heisterkamp N et al (1986) The chronic myelocytic cell line K562 contains a breakpoint in bcr and produces a chimeric bcr/c-abl transcript. Mol Cell Biol 6(2):607–616
209. Deininger MW, Goldman JM, Melo JV (2000) The molecular biology of chronic myeloid leukemia. Blood 96(10):3343–3356
210. Daley GQ, Baltimore D (1988) Transformation of an interleukin 3-dependent hematopoietic cell line by the chronic myelogenous leukemia-specific P210bcr/abl protein. Proc Natl Acad Sci U S A 85(23):9312–9316
211. Elefanty AG, Hariharan IK, Cory S (1990) bcr-abl, the hallmark of chronic myeloid leukaemia in man, induces multiple haemopoietic neoplasms in mice. EMBO J 9(4):1069–1078
212. Gishizky ML, Johnson-White J, Witte ON (1993) Efficient transplantation of BCR-ABL-induced chronic myelogenous leukemia-like syndrome in mice. Proc Natl Acad Sci U S A 90(8):3755–3759
213. Kelliher M, Knott A, McLaughlin J, Witte ON, Rosenberg N (1991) Differences in oncogenic potency but not target cell specificity distinguish the two forms of the BCR/ABL oncogene. Mol Cell Biol 11(9):4710–4716
214. Li S, Ilaria RL Jr, Million RP, Daley GQ, Van Etten RA (1999) The P190, P210, and P230 forms of the BCR/ABL oncogene induce a similar chronic myeloid leukemia-like syndrome in mice but have different lymphoid leukemogenic activity. J Exp Med 189(9):1399–1412
215. Bernt KM, Hunger SP (2014) Current concepts in pediatric Philadelphia chromosome-positive acute lymphoblastic leukemia. Front Oncol 4:54. Epub 2014/04/12
216. Hantschel O (2012) Structure, regulation, signaling, and targeting of abl kinases in cancer. Genes Cancer 3(5–6):436–446. Epub 2012/12/12
217. Ilaria RL Jr, Van Etten RA (1995) The SH2 domain of P210BCR/ABL is not required for the transformation of hematopoietic factor-dependent cells. Blood 86(10):3897–3904
218. Lugo TG, Pendergast AM, Muller AJ, Witte ON (1990) Tyrosine kinase activity and transformation potency of bcr-abl oncogene products. Science 247(4946):1079–1082
219. Wetzler M, Talpaz M, Van Etten RA, Hirsh-Ginsberg C, Beran M, Kurzrock R (1993) Subcellular localization of Bcr, Abl, and Bcr-Abl proteins in normal and leukemic cells and correlation of expression with myeloid differentiation. J Clin Invest 92(4):1925–1939. Epub 1993/10/01
220. Van Etten RA, Jackson P, Baltimore D (1989) The mouse type IV c-abl gene product is a nuclear protein, and activation of transforming ability is associated with cytoplasmic localization. Cell 58(4):669–678
221. Melo JV, Deininger MW (2004) Biology of chronic myelogenous leukemia–signaling pathways of initiation and transformation. Hematol Oncol Clin North Am 18(3):545–568, vii–viii
222. Asmussen J, Lasater EA, Tajon C, Oses-Prieto J, Jun YW, Taylor BS et al (2014) MEK-dependent negative feedback underlies BCR-ABL-mediated oncogene addiction. Cancer Discov 4(2):200–215
223. Notari M, Neviani P, Santhanam R, Blaser BW, Chang JS, Galietta A et al (2006) A MAPK/HNRPK pathway controls BCR/ABL oncogenic potential by regulating MYC mRNA translation. Blood 107(6):2507–2516

224. Cortez D, Reuther G, Pendergast AM (1997) The Bcr-Abl tyrosine kinase activates mitogenic signaling pathways and stimulates G1-to-S phase transition in hematopoietic cells. Oncogene 15(19):2333–2342
225. Varticovski L, Daley GQ, Jackson P, Baltimore D, Cantley LC (1991) Activation of phosphatidylinositol 3-kinase in cells expressing abl oncogene variants. Mol Cell Biol 11(2):1107–1113
226. Reuther JY, Reuther GW, Cortez D, Pendergast AM, Baldwin AS Jr (1998) A requirement for NF-kappaB activation in Bcr-Abl-mediated transformation. Genes Dev 12(7):968–981
227. Carlesso N, Frank DA, Griffin JD (1996) Tyrosyl phosphorylation and DNA binding activity of signal transducers and activators of transcription (STAT) proteins in hematopoietic cell lines transformed by Bcr/Abl. J Exp Med 183(3):811–820
228. Raitano AB, Halpern JR, Hambuch TM, Sawyers CL (1995) The Bcr-Abl leukemia oncogene activates Jun kinase and requires Jun for transformation. Proc Natl Acad Sci U S A 92(25):11746–11750
229. Sawyers CL, McLaughlin J, Witte ON (1995) Genetic requirement for Ras in the transformation of fibroblasts and hematopoietic cells by the Bcr-Abl oncogene. J Exp Med 181(1):307–313
230. Skorski T, Kanakaraj P, Nieborowska-Skorska M, Ratajczak MZ, Wen SC, Zon G et al (1995) Phosphatidylinositol-3 kinase activity is regulated by BCR/ABL and is required for the growth of Philadelphia chromosome-positive cells. Blood 86(2):726–736
231. Skorski T, Bellacosa A, Nieborowska-Skorska M, Majewski M, Martinez R, Choi JK et al (1997) Transformation of hematopoietic cells by BCR/ABL requires activation of a PI-3k/Akt-dependent pathway. EMBO J 16(20):6151–6161
232. Nieborowska-Skorska M, Wasik MA, Slupianek A, Salomoni P, Kitamura T, Calabretta B et al (1999) Signal transducer and activator of transcription (STAT)5 activation by BCR/ABL is dependent on intact Src homology (SH)3 and SH2 domains of BCR/ABL and is required for leukemogenesis. J Exp Med 189(8):1229–1242. Epub 1999/04/20
233. Mullighan CG, Miller CB, Radtke I, Phillips LA, Dalton J, Ma J et al (2008) BCR-ABL1 lymphoblastic leukaemia is characterized by the deletion of Ikaros. Nature 453(7191):110–114. Epub 2008/04/15
234. Ribeiro RC, Abromowitch M, Raimondi SC, Murphy SB, Behm F, Williams DL (1987) Clinical and biologic hallmarks of the Philadelphia chromosome in childhood acute lymphoblastic leukemia. Blood 70(4):948–953
235. Crist W, Carroll A, Shuster J, Jackson J, Head D, Borowitz M et al (1990) Philadelphia chromosome positive childhood acute lymphoblastic leukemia: clinical and cytogenetic characteristics and treatment outcome. A Pediatric Oncology Group study. Blood 76(3):489–494
236. Fletcher JA, Lynch EA, Kimball VM, Donnelly M, Tantravahi R, Sallan SE (1991) Translocation (9;22) is associated with extremely poor prognosis in intensively treated children with acute lymphoblastic leukemia. Blood 77(3):435–439
237. Kusio-Kobialka M, Wolanin K, Podszywalow-Bartnicka P, Sikora E, Skowronek K, McKenna SL et al (2012) Increased acetylation of lysine 317/320 of p53 caused by BCR-ABL protects from cytoplasmic translocation of p53 and mitochondria-dependent apoptosis in response to DNA damage. Apoptosis Int J Program Cell Death 17(9):950–963
238. Daflon-Yunes N, Pinto-Silva FE, Vidal RS, Novis BF, Berguetti T, Lopes RR et al (2013) Characterization of a multidrug-resistant chronic myeloid leukemia cell line presenting multiple resistance mechanisms. Mol Cell Biochem 383(1-2):123–135
239. Skorski T (2002) BCR/ABL regulates response to DNA damage: the role in resistance to genotoxic treatment and in genomic instability. Oncogene 21(56):8591–8604
240. von Bubnoff N, Peschel C, Duyster J (2003) Resistance of Philadelphia-chromosome positive leukemia towards the kinase inhibitor imatinib (STI571, Glivec): a targeted oncoprotein strikes back. Leukemia 17(5):829–838
241. Gorre ME, Sawyers CL (2002) Molecular mechanisms of resistance to STI571 in chronic myeloid leukemia. Curr Opin Hematol 9(4):303–307

242. Schultz KR, Bowman WP, Aledo A, Slayton WB, Sather H, Devidas M et al (2009) Improved early event-free survival with imatinib in Philadelphia chromosome-positive acute lymphoblastic leukemia: a children's oncology group study. J Clin Oncol Off J Am Soc Clin Oncol 27(31):5175–5181. Epub 2009/10/07
243. Karaman MW, Herrgard S, Treiber DK, Gallant P, Atteridge CE, Campbell BT et al (2008) A quantitative analysis of kinase inhibitor selectivity. Nat Biotechnol 26(1):127–132. Epub 2008/01/10
244. Wilson MB, Schreiner SJ, Choi HJ, Kamens J, Smithgall TE (2002) Selective pyrrolopyrimidine inhibitors reveal a necessary role for Src family kinases in Bcr-Abl signal transduction and oncogenesis. Oncogene 21(53):8075–8088. Epub 2002/11/22
245. Lilly MB, Ottmann OG, Shah NP, Larson RA, Reiffers JJ, Ehninger G et al (2010) Dasatinib 140 mg once daily versus 70 mg twice daily in patients with Ph-positive acute lymphoblastic leukemia who failed imatinib: results from a phase 3 study. Am J Hematol 85(3):164–170. Epub 2010/02/05
246. Ottmann O, Dombret H, Martinelli G, Simonsson B, Guilhot F, Larson RA et al (2007) Dasatinib induces rapid hematologic and cytogenetic responses in adult patients with Philadelphia chromosome positive acute lymphoblastic leukemia with resistance or intolerance to imatinib: interim results of a phase 2 study. Blood 110(7):2309–2315. Epub 2007/05/15
247. Kalman L, Lindegren ML, Kobrynski L, Vogt R, Hannon H, Howard JT et al (2004) Mutations in genes required for T-cell development: IL7R, CD45, IL2RG, JAK3, RAG1, RAG2, ARTEMIS, and ADA and severe combined immunodeficiency: HuGE review. Genet Med Off J Am Coll Med Genet 6(1):16–26. Epub 2004/01/17
248. Higuchi M, Asao H, Tanaka N, Oda K, Takeshita T, Sugamura K (1997) Regulation of IL-2 signaling. Leukemia 11(Suppl 3):416–417
249. Igaz P, Toth S, Falus A (2001) Biological and clinical significance of the JAK-STAT pathway; lessons from knockout mice. Inflamm Res Off J Eur Histamine Res Soc [et al] 50(9):435–441
250. Roberts KG, Morin RD, Zhang J, Hirst M, Zhao Y, Su X et al (2012) Genetic alterations activating kinase and cytokine receptor signaling in high-risk acute lymphoblastic leukemia. Cancer Cell 22(2):153–166
251. Bandapalli OR, Schuessele S, Kunz JB, Rausch T, Stutz AM, Tal N et al (2014) The activating STAT5B N642H mutation is a common abnormality in pediatric T-cell acute lymphoblastic leukemia and confers a higher risk of relapse. Haematologica 99:e188–e192
252. Yoda A, Yoda Y, Chiaretti S, Bar-Natan M, Mani K, Rodig SJ et al (2010) Functional screening identifies CRLF2 in precursor B-cell acute lymphoblastic leukemia. Proc Natl Acad Sci U S A 107(1):252–257. Epub 2009/12/19
253. Mullighan CG, Collins-Underwood JR, Phillips LA, Loudin MG, Liu W, Zhang J et al (2009) Rearrangement of CRLF2 in B-progenitor- and Down syndrome-associated acute lymphoblastic leukemia. Nat Genet 41(11):1243–1246. Epub 2009/10/20
254. Mansour MR, Reed C, Eisenberg AR, Tseng JC, Twizere JC, Daakour S, Yoda A, Rodig SJ, Tal N, Shochat C, Berezovskaya A, DeAngelo DJ, Sallan SE, Weinstock DM, Izraeli S, Kung AL, Kentsis A, Look AT (2015) Targeting oncogenic interleukin-7 receptor signalling with N-acetylcysteine in T cell acute lymphoblastic leukaemia. Br J Haematol 168(2):230–238
255. Attarbaschi A, Morak M, Cario G, Cazzaniga G, Ensor HM, te Kronnie T et al (2012) Treatment outcome of CRLF2-rearranged childhood acute lymphoblastic leukaemia: a comparative analysis of the AIEOP-BFM and UK NCRI-CCLG study groups. Br J Haematol 158(6):772–777
256. Cario G, Zimmermann M, Romey R, Gesk S, Vater I, Harbott J et al (2010) Presence of the P2RY8-CRLF2 rearrangement is associated with a poor prognosis in non-high-risk precursor B-cell acute lymphoblastic leukemia in children treated according to the ALL-BFM 2000 protocol. Blood 115(26):5393–5397

257. Chen IM, Harvey RC, Mullighan CG, Gastier-Foster J, Wharton W, Kang H et al (2012) Outcome modeling with CRLF2, IKZF1, JAK, and minimal residual disease in pediatric acute lymphoblastic leukemia: a Children's Oncology Group study. Blood 119(15):3512–3522
258. van der Veer A, Waanders E, Pieters R, Willemse ME, Van Reijmersdal SV, Russell LJ et al (2013) Independent prognostic value of BCR-ABL1-like signature and IKZF1 deletion, but not high CRLF2 expression, in children with B-cell precursor ALL. Blood 122(15):2622–2629
259. Shochat C, Tal N, Bandapalli OR, Palmi C, Ganmore I, te Kronnie G et al (2011) Gain-of-function mutations in interleukin-7 receptor-alpha (IL7R) in childhood acute lymphoblastic leukemias. J Exp Med 208(5):901–908. Epub 2011/05/04
260. Zenatti PP, Ribeiro D, Li W, Zuurbier L, Silva MC, Paganin M et al (2011) Oncogenic IL7R gain-of-function mutations in childhood T-cell acute lymphoblastic leukemia. Nat Genet 43(10):932–939. Epub 2011/09/06
261. Porcu M, Kleppe M, Gianfelici V, Geerdens E, De Keersmaecker K, Tartaglia M et al (2012) Mutation of the receptor tyrosine phosphatase PTPRC (CD45) in T-cell acute lymphoblastic leukemia. Blood 119(19):4476–4479. Epub 2012/03/23
262. Treanor LM, Zhou S, Janke L, Churchman ML, Ma Z, Lu T et al (2014) Interleukin-7 receptor mutants initiate early T cell precursor leukemia in murine thymocyte progenitors with multipotent potential. J Exp Med 211(4):701–713
263. Graux C, Cools J, Melotte C, Quentmeier H, Ferrando A, Levine R et al (2004) Fusion of NUP214 to ABL1 on amplified episomes in T-cell acute lymphoblastic leukemia. Nat Genet 36(10):1084–1089
264. De Keersmaecker K, Rocnik JL, Bernad R, Lee BH, Leeman D, Gielen O et al (2008) Kinase activation and transformation by NUP214-ABL1 is dependent on the context of the nuclear pore. Mol Cell 31(1):134–142. Epub 2008/07/11
265. Hagemeijer A, Graux C (2010) ABL1 rearrangements in T-cell acute lymphoblastic leukemia. Genes Chromosomes Cancer 49(4):299–308
266. Stergianou K, Fox C, Russell NH (2005) Fusion of NUP214 to ABL1 on amplified episomes in T-ALL–implications for treatment. Leukemia 19(9):1680–1681. Epub 2005/07/15
267. Deenik W, Beverloo HB, van der Poel-van de Luytgaarde SC, Wattel MM, van Esser JW, Valk PJ et al (2009) Rapid complete cytogenetic remission after upfront dasatinib monotherapy in a patient with a NUP214-ABL1-positive T-cell acute lymphoblastic leukemia. Leukemia 23(3):627–629. Epub 2008/11/07
268. Stam RW, den Boer ML, Schneider P, Nollau P, Horstmann M, Beverloo HB et al (2005) Targeting FLT3 in primary MLL-gene-rearranged infant acute lymphoblastic leukemia. Blood 106(7):2484–2490
269. Brown P, Levis M, Shurtleff S, Campana D, Downing J, Small D (2005) FLT3 inhibition selectively kills childhood acute lymphoblastic leukemia cells with high levels of FLT3 expression. Blood 105(2):812–820
270. Roberts KG, Li Y, Payne-Turner D, Harvey RC, Yang YL, Pei D et al (2014) Targetable kinase-activating lesions in Ph-like acute lymphoblastic leukemia. N Engl J Med 371(11):1005–1015. Epub 2014/09/11
271. Graux C, Stevens-Kroef M, Lafage M, Dastugue N, Harrison CJ, Mugneret F et al (2009) Heterogeneous patterns of amplification of the NUP214-ABL1 fusion gene in T-cell acute lymphoblastic leukemia. Leukemia 23(1):125–133. Epub 2008/10/17
272. Daigle SR, Olhava EJ, Therkelsen CA, Majer CR, Sneeringer CJ, Song J et al (2011) Selective killing of mixed lineage leukemia cells by a potent small-molecule DOT1L inhibitor. Cancer Cell 20(1):53–65. Epub 2011/07/12
273. Harris WJ, Huang X, Lynch JT, Spencer GJ, Hitchin JR, Li Y et al (2012) The histone demethylase KDM1A sustains the oncogenic potential of MLL-AF9 leukemia stem cells. Cancer Cell 21(4):473–487
274. Placke T, Faber K, Nonami A, Putwain SL, Salih HR, Heidel FH et al (2014) Requirement for CDK6 in MLL-rearranged acute myeloid leukemia. Blood 124:13–23

Chapter 11
Cellular and Molecular Basis of *KMT2A/MLL* Leukaemias: From Transformation Mechanisms to Novel Therapeutic Strategies

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Contents

11.1 Introduction 224
11.2 11q23 Abnormalities in Acute Leukaemia 225
11.3 *MLL* Chromosomal Translocation Fusions 226
11.4 MLL-Partial Tandem Duplication (MLL-PTD) 228
11.5 MLL Amplification and Internal Deletion 229
11.6 Modelling *MLL* Leukaemia 229
11.6.1 AML Models of *MLL* Fusions 229
11.6.2 ALL Models of MLL Fusions 230
11.6.3 ABL Generated by MLL Fusion 231
11.7 Cell of Origin for MLL Leukaemia 231
11.8 Transcription Regulation by MLL and MLL Fusions During Normal and Disease Development 232
11.8.1 Wild-Type MLL and Its Transcriptional Complex 232
11.8.2 MLL Fusion Complexes 234
11.8.3 Crosstalk Between Wild-Type MLL and MLL Fusion Complexes 234
11.9 Pathways and Downstream Targets Critical in MLL Leukaemia 235
11.9.1 MLL Downstream Targets 236
11.9.2 Critical Pathways/Molecules Not Directly Regulated by MLL Fusions 237
11.9.2.1 Canonical WNT/beta-catenin 237
11.9.2.2 ITGB3 237
11.9.2.3 Polycomb Group Proteins 237
11.9.2.4 RUNX1 238
11.9.2.5 Other Emerging Molecules 238
11.10 Current Therapies and Development of Novel Targeted Therapies 239
11.10.1 Targets with Enzymatic Activities 239
11.10.1.1 DOT1L 239
11.10.1.2 PRMT1. 241
11.10.1.3 KDM1A/LSD1 241

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11.10.2 Targeting the Protein-Protein Interactions 241
11.10.2.1 BRD4 241
11.10.2.2 MEN1 242
11.10.2.3 WDR5. 243
11.10.2.4 PAF1/PAFc 243
11.11 Concluding Remarks and Perspective 243
11.12 Appendix 244
Acknowledgements 244
References 244

Abstract The Mixed Lineage Leukaemia (KMT2A/MLL) gene encoding a H3K4me3 histone methyltransferase is a frequent target of chromosomal aberrations resulting in various forms of aggressive leukaemia with poor prognosis. Treatment of MLL leukaemia presents a major challenge, thus novel and more effective therapies are urgently needed to improve patients' outlook. While internal deletion and amplification of MLL have been reported, MLL mutations mostly manifest either as chromosomal translocations resulting in the generation of fusion proteins, in which the C-terminus of MLL is replaced by 1 of more than 70 identified fusion partners, or as partial internal tandem duplications (PTD). Some of the most frequent MLL fusion partners exist in multiple complexes associated with histone methyltransferase, DOT1L or positive elongation factor b (P-TEFb). Aberrant recruitment of these complexes by MLL fusions among other mechanisms such as dimerization of MLL fusions or recruitment of other histone modifying enzymes resulting in aberrant transcription of downstream targets such as *HOX* genes has been identified as critical steps in MLL fusion mediated transformation. Among them are various key components of epigenetic machinery including DOT1L, PRMT1 and BRD4, which emerge as promising therapeutic targets. On the other hand, recent studies also identified other essential pathways and molecules such as beta-catenin, ITGB3/SYK, polycomb proteins that are not necessarily under the direct control of the MLL fusions. While development of small molecule inhibitors against most of these emerging targets is still in very early stages, the latest development of DOT1L inhibitors currently in a phase I clinical trial on MLL leukaemia demonstrates the promise of translating our knowledge into novel treatments to improve the outcome for *MLL* leukaemia patients.

Keywords Acute leukaemia • KMT2A/MLL leukaemia stem cells • Cell of origin • Epigenetics • Targeted therapy

11.1 Introduction

Acute leukaemia is characterized by rapid expansion of immature white blood cells that accumulate in the bone marrow and interfere with the production of normal blood cells. Clinical classification based on lineage characteristics of the leukaemic

blasts can broadly divide acute leukaemia into acute myeloid leukaemia (AML), acute lymphoid leukaemia (ALL) and acute biphenotypic leukaemia (ABL), which features markers of both myeloid and lymphoid cells. While ALL is the most common cancer in infants and children, ABL uniformly present at all ages whereas the incidence of AML increases with age. Recurring chromosomal aberrations tend to associate with particular subtype of acute leukaemia, e.g., t(15;17) with *PML-RARA*, t(8;21) with *RUNX1-RUNX1T1*/*AML1-ETO* found only in AML; whereas t(1;19) encoding *TCF3*/*E2A-PBX1* only occurs in ALL. Interestingly, 11q23 chromosomal aberrations involving the *KMT2A*/*MLL* (*mixed lineage leukaemia*) gene, which generally confer very poor prognosis, are found promiscuously in ALL [1], AML [2] and ABL [3]. *MLL* rearrangement can be found in up to 80 % in infant leukaemia, 3–10 % in ALL and AML, and up to 18 % in ABL. The *MLL* gene located at 11q23 is the human homolog of *drosophila trithorax* (*trx*), and encodes a SET-domain histone methyltransferase (HMT) that tri-methylates histone 3 lysine 4 (H3K4me3) positively associated with transcription. 430 kDa MLL protein also contains N-terminal DNA binding domains (AT-hooks and CXXC domain) as well as central PHD fingers and a transactivation domain. MLL protein is proteolytically cleaved between PHD fingers and the transactivation domain into two fragments (MLL-N and MLL-C), which specifically associate via consensus interaction motifs to regulate gene expression for normal development, including haematopoiesis. In contrast, its mutations in the haematopoietic system lead to acute leukaemia. In this book chapter, we will review the recent advance in understanding the cellular and molecular basis of MLL leukaemia. We will discuss existing *MLL* leukaemia models, the potential cell of origin of MLL leukaemia, compare the molecular functions of wild-type MLL with oncogenic MLL fusions, highlight critical pathways/molecules in MLL leukaemia, and finally describe current therapies and potential development of novel targeted therapies for MLL leukaemia.

11.2 11q23 Abnormalities in Acute Leukaemia

Four recurrent 11q23 chromosomal abnormalities have been identified in acute leukaemia, namely chromosomal translocations, partial tandem duplication (PTD), amplification and internal deletion (Fig. 11.1).

While *MLL*-PTD, amplification and deletion can be found in AML, *MLL* translocations remain the most recurrent 11q23 abnormality in all different acute leukaemia subtypes. The treatments of leukaemia with 11q23 aberrations remain a major clinical challenge. Although cure rates of up to 80 % can be achieved for non-11q23 childhood ALL, the outlook for patients with 11q23 abnormalities is far worse [4]. Similarly, the presence of 11q23 translocations generally confers poor prognosis in AML [2] and in ABL [3].

Fig. 11.1 Schematic overview of wild-type KMT2A/MLL protein and the aberrant KMT2A/MLL proteins resulting from different 11q23 chromosomal aberrations. The incidence of the individual 11q23 aberration amongst all 11q23 re-arrangements in infant/childhood and adult ALL and AML are indicated (Data modified from [7]). All 'rare' MLL translocation partners are presented together in 'all others'. Functional protein domains of MLL are indicated. AT-hooks and CXXC, DNA binding domains; FRYN/FRYC, consensus interaction motifs; TAD, transactivation domain; SET domain, mediates H3K4me1,2,3 methylation

11.3 *MLL* Chromosomal Translocation Fusions

Pre-natal backtracking of concordant leukaemia in identical monozygotic twins provided evidence that 11q23 translocations can occur in utero [5], and the very short latency (<1 year) to develop full blown leukaemia in infants suggest few (if any) additional mutations are required. This may differ from the aetiology of leukaemia harbouring 11q23 translocations found in adults where the cellular origin of the disease can be very different. On the other hand, *MLL* translocations not only occur in de novo acute leukaemia, but are also frequently observed in therapy related AML (t-AML). Strikingly, compared to de novo acute leukaemia where 11q23 translocations occur in similar frequencies in AML and ALL, the vast majority (>90 %) of 11q23 translocation therapy related leukaemia is t-AML, that arise after topoisomerase II treatment of an unrelated primary neoplasm [6].

All *MLL* translocations occur within the same 8.3 kb breakpoint cluster region between the CXXC DNA-binding domain and the central PHD fingers. As a result, all MLL fusions retain the MLL N-terminus with its DNA binding motifs, but the C-terminal SET domain is replaced with the fusion partner that may possess other transcriptional effector or homo-dimerization domains. Although more than 70 different MLL fusion partners have been identified and can be broadly divided into nuclear and cytoplasmic proteins, AFF1/AF4, MLLT3/AF9, MLLT10/AF10, ELL, MLLT1/ENL and MLLT4/AF6 account for more than 85 % of cases [7]. With the exception of AF6, they are all nuclear proteins. Strikingly, the most frequent nuclear fusion partners biochemically interact with each other [8], and many of them were subsequently found co-existing in protein complexes. The first description of such a complex was named ENL associated proteins (EAP) [9, 10] (Fig. 11.2), which included amongst other components also known MLL fusion partners ENL, AF9, AF4, AFF3/LAF4, AFF4/AF5Q31 as well as the H3K79 histone methyltransferase

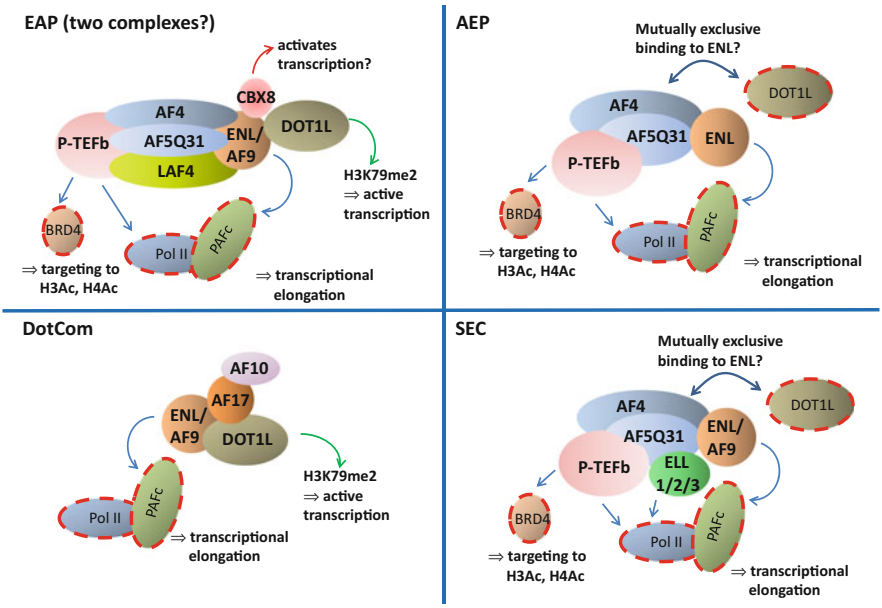

Fig. 11.2 Schematic overview of the complexes associated with the most common KMT2A/MLL fusion partners. *EAP* ENL associated proteins, *AEP* AF4 family/ENL family/P-TEFb, *DotCom* Dot1L complex, *SEC* super elongation complex. Proteins not originally identified in these complexes but shown to interact with components of these complexes, indicated with *blue arrows*, are marked with a *dotted red outline. Green arrows* indicate H3K79me2 chromatin modification mediated by Dot1L, whereas *red arrow* indicates potential positive transcriptional effects. BRD4 recognizes and binds to acetylated histones and interacts with P-TEF-b. PAF1 promotes transcriptional elongation of RNA polymerase II (Pol II) and interacts with ENL/AF9. Pol II itself interacts with P-TEFb and ELL/ELL2/ELL3. A mutually exclusive binding of ENL to either AF4 or DOT1L was suggested [12]

DOT1L and the positive transcription elongation factor b (P-TEFb) that plays an essential role in regulation of the transcription by RNA polymerase II.

However, more recent biochemical and functional studies have identified three slightly different complexes, namely the super elongation complex (SEC) [11], AF4 family/ENL family/P-TEFb (AEP) complex [12] and DotCom [13]. While AEP and SEC largely overlap and contain P-TEFb as well as known MLL fusion partners AF4, AF5Q31 and ENL (note that SEC also contained ELL1/ELL2/ELL3 and AF9), they do not have DOT1L. On the other hand, DotCom lacks P-TEFb but contains DOT1L plus MLLT3/AF9, AF10, MLLT6/AF17 and ENL. These results suggest the presence of at least two different complexes (one with and one without DOT1L) associated with the most common nuclear MLL fusion partners (Fig. 11.2) that may link transcriptional elongation with histone methylation leading to deregulated target gene expression, such as *HOX* genes. Interestingly, while the incidence of the different MLL fusion partners in ALL as well as AML changes with the age of the patients (Fig. 11.1), certain MLL fusions are almost exclusively associated with AML (MLL-ELL) or ALL (MLL-AF4) whereas others can be found in both (MLL-AF9, MLL-ENL). Therefore, some MLL fusions may in part determine the lineage specificity, although other factors including cell of origin and microenvironments likely also have important roles in controlling the MLL leukaemia phenotypes. On the other hand, given that transcriptional complexes associated with an individual MLL fusion are likely similar in AML and ALL, these findings suggest a lack of functional relationship between transcriptional complexes and the leukaemia lineage.

11.4 MLL-Partial Tandem Duplication (MLL-PTD)

MLL-PTD occurs within the same breakpoint cluster region observed in *MLL* translocations, but results in an internal duplication of N-terminal sequences flanking the DNA binding domains AT hooks and CXXC. Importantly, this re-arrangement of MLL does not affect C-terminal sequences of MLL. Therefore MLL-PTD possesses a functional H3K4me3 SET domain and has been reported with strong transcriptional activity [14]. MLL-PTD predominantly occur in AML (~5 %) and is enriched in AML with trisomy 11 [2], although very rare cases in ALL have been reported [7]. While MLL-PTD enhances self-renewal of haematopoietic progenitor cells, it does not induce AML in mouse model, suggesting additional mutations are required for full-blown leukaemia [15–17]. Similar to *MLL* translocations, the presence of *MLL*-PTD in AML confers a poor prognosis with markedly reduced remission duration [18].

11.5 MLL Amplification and Internal Deletion

In contrast to *MLL* translocation and *MLL*-PTD, *MLL* amplification and internal deletion are much less frequent 11q23 aberrations. *MLL* amplification is mainly the result of intrachromosomal (HSR, homogenous staining regions) or extrachromosomal (dmin, double minute chromosome) amplifications, and may confer poor prognosis [19]. *MLL* amplification is predominately found in AML (up to 1 %), but a case report of its existence in ALL has also been described [20]. On the other hand, only three ALL patients have been reported with internal deletion in one *MLL* allele, in which exon 11 together with parts of intron 10 and 11 (new nomenclature) were lost [19]. Since *MLL* translocations represent the most frequent 11q23 abnormalities in acute leukaemia and *MLL*-PTD/amplification has been recently reviewed [19], we will focus our discussion on *MLL* translocations in the rest of the chapter.

11.6 Modelling *MLL* Leukaemia

11.6.1 AML Models of MLL *Fusions*

In the past two decades, a number of *MLL* leukaemia models have been reported that recapitulate several aspects of the human disease. A breakthrough in modelling *MLL* fusions came around 4–5 years after the identification [21] and cloning of *MLL* gene at 11q23 [22, 23] when Rabbitts lab [24] created the first *MLL* fusion mouse model. Using knock-in technologies in ES cells, the cDNA of the fusion partner AF9 was inserted just after the exon 8 (old nomenclature) of the *MLL* gene resulting in an MLL-fusion gene under the expression of the endogenous *MLL* promoter. Chimeric MLL-AF9 knock-in mice developed AML with a latency of 4–11 month. The second seminal *MLL* leukaemia model was established by the Cleary lab using retroviral transduction to transfer MLL-ENL into murine primary haematopoietic progenitor cells. MLL-ENL expressing murine primary haematopoietic cells induced myeloid leukaemia when transplanted into mice with a shorter latency of 2–5 months [25]. This system has also been used to define and describe the disease progression from pre-LSC (pre-leukaemic stem cell) to LSC [2, 26]. In order to mimic and recreate the chromosomal translocations found in human acute leukaemia patients rather than just the expression of the dominant der(11) transcripts, the Rabbitts lab further pioneered in the generation of the translocator mice [27]. These mice harboured loxp sites within the Mll and Af9 genes at specific introns, which correspond to the breakpoint regions found in MLL-AF9 acute leukaemia patients. Exposure to *Cre*-recombinase resulted in the interchromosomal recombination of Mll and Af9, thus creating both derivative transcripts and resulting in AML [28]. A similar approach was also used in Mll-Enl translocator mice that rapidly developed myeloid leukaemia after *Cre*-recombination [29] with latencies similar to that

observed in the retroviral transduction model. The use of a conditional *MLL* fusion by employing either the mutant oestrogen receptor/tamoxifen [30] or Tet off/doxycycline system [31] together with retroviral transduction of murine primary cells has further demonstrated the biological relevance of MLL fusions and their downstream targets such as *HOX*/*MEIS* genes in MLL leukaemia. Modified retroviral transduction approaches using MLL fusions and human lineage negative or CD34+ primary haematopoietic cells isolated from cord blood led to the first humanized in vivo leukaemia model [32, 33]. Together, these landmark studies demonstrated the feasibility of modelling AML as a result of 11q23 translocations/fusions, and these models have been widely used to gain mechanistic insights into the underlying disease mechanisms. However, modelling the ALL phenotype observed in 11q23 patients proved to be more challenging.

11.6.2 ALL Models of MLL Fusions

The first ALL model of MLL leukaemia was reported in a murine retroviral transduction approach using *MLL-GAS7*, which was capable of transforming HSCs leading to multiple lineage leukaemia including ALL, AML and ABL [34]. *Mll-Enl* translocator mice developed T-ALL among other haematological malignancies when *Cre* expression was restricted to the t-cell compartment using a *Lck-Cre* [28]. On the other hand, immuno-compromised NOD/SCID mice transplanted with *MLL-ENL* or *MLL-AF9* transduced human primary cells developed ALL, or ALL/AML respectively [33]. Interestingly, the phenotype of leukaemia seems to depend largely on the microenvironment of the recipient mice as *MLL-AF9* transduced human CD34+ cells have shown different disease outcomes, ALL vs AML, depending on the recipient mouse strain [32]. Modelling the ALL disease phenotype of *MLL-AF4*, the most frequent *MLL* fusion associated with ALL has also been challenging. Early unsuccessful attempts to model *MLL-AF4* in murine primary cells using a retroviral approach were succeeded by two mouse models (knock-in and inverter), in which *MLL-AF4* expression led to B-cell lymphomas with long latency and low penetrance [35–37]. A much improved conditional *MLL-AF4* model was reported to have about half of the knock-in mice develop B-ALL [38]. However, the observed ALL phenotype was still different from that observed in patients and the other half of the animals developed AML, suggesting that the right cellular target might have been missed in these studies [39]. Of note, another *MLL-AF4* murine retroviral transduction model has been reported, in which *MLL-AF4* as well as the reciprocal translocation product *AF4-MLL* causes B-ALL with a long latency and low penetrance [40]. Surprisingly, this study also showed that *AF4-MLL* alone is sufficient to cause leukaemia, suggesting a critical role for the reciprocal product in the pathogenesis of *MLL-AF4* leukaemia. However, it should be noted that the

reciprocal MLL fusions, including AF4-MLL cannot be detected in all patients harbouring *MLL* translocations [7, 41, 42].

11.6.3 ABL Generated by MLL Fusion

While ABL comprises only 2–5 % of all acute leukaemia cases [3], bi-phenotypic leukaemia expressing MLL fusions have been frequently described in different model systems. The first ABL models were reported using retroviral transduction approach on murine cells with *MLL-ENL* [43] or *MLL-GAS7* [34]. In both cases, *MLL fusion* transformed cells expressed both myeloid and lymphoid markers, and induced ABL in mice with a relative short latency. ABL was also observed in three *MLL-AF4* mice [38] and one *AF4-MLL* mouse [40], respectively, Interestingly, ABL was recurrently observed in the retroviral transduction approach with human CD34+ primary haematopoietic cells isolated from cord blood [32]. These results provide further experimental evidence for the specific association of *MLL* fusions with lineage infidelity/promiscuity.

11.7 Cell of Origin for MLL Leukaemia

In order to define potential origins of MLL LSCs, phenotypically and functionally defined haematopoietic populations (such as HSC, haematopoietic stem cell; MPP, multipotent progenitor; CMP, common myeloid progenitor; GMP, granulocyte-macrophage progenitor; MEP, megakaryocyte erythroid progenitor) were purified from mouse bone marrow for retroviral transduction and transformation assays. While MLL-ENL failed to transform MEP, it induced phenotypically identical myeloid leukaemia when expressed in HSC, CMP and GMP populations [44]. In contrast, MLL-GAS7 was reported to transform HSC, CMP and GMP but with different leukaemia phenotypes. MLL-GAS7 expression in HSCs could produce multi-lineage leukaemia whereas its expression in CMP and GMP led to exclusive myeloid transformation [34]. These results together with subsequent global expression analyses on MLL LSC-enriched populations [45, 46] revealed that MLL fusion can induce a self-renewal programme in otherwise short-lived myeloid progenitor cells in AML, whereas HSCs may be the cell of origin for multi-lineage leukaemia. On the other hand, an important insight about the potential origin of LSC was obtained in a study examining the transformation abilities of HSCs, CMPs and GMPs expressing MLL-AF9 driven by endogenous *MLL* promoter in *MLL-AF9* knock-in mice. While *MLL-AF9* expressing HSCs led to AML, GMPs expressing

MLL-AF9 driven by the endogenous *MLL* promoter, which is in contrast to those driven by MSCV retroviral promoter [45], failed to induce leukaemia, highlighting the importance of the expression level in determining the transformation ability of MLL fusions in different cellular targets [47]. Although it is feasible to compare murine retroviral and knock-in models, it is more challenging to assess the impact on MLL fusion expression levels in the human system. Recent technological advances in genome editing technologies utilizing custom made zinc finger nucleases (ZFNs) [48] or transcriptional activator-like effector nucleases (TALENs) [49] that recognize any given DNA sequence with high specificity can facilitate the creation of novel MLL leukaemia models in human primary cells where the MLL fusion is expressed at physiological levels. Such a model system could also provide unique insights into the long sought-after infant ALL leukaemia with an extremely brief latency. Given that MLL fusions arise in utero, it would be important to assess the impact of physiologically expressed MLL fusion in early, embryonic haematopoiesis if hES or iPS cell are employed.

11.8 Transcription Regulation by MLL and MLL Fusions During Normal and Disease Development

11.8.1 Wild-Type MLL and Its Transcriptional Complex

It had been realized very early on that the *MLL* gene shares significant homology with the drosophila trithorax (*trx*) gene [22]. Trx is the founding member of the trithorax-group (Trx-G) proteins, which antagonize the polycomb group (Pc-G) proteins to maintain cellular memory/identity by epigenetically maintaining information about gene expression of key developmental master regulators such as hox genes [50]. Consistently, *MLL* knockout mice were embryonic lethal and showed homeotic transformations with abnormal *Hox* gene expression [51], indicating MLL as the functional homolog of trx with critical roles in cellular memory and the transcriptional regulation. Important cues to the molecular function of MLL came from the discovery that MLL is the mammalian homolog to yeast set1 [52], which was later shown to possess histone methyltransferase activity specific for H3K4 [53]. Subsequently, this enzymatic activity was also confirmed in MLL [54] and Trx [55]. The H3K4me3 mark is generally associated with active transcription (active mark) although it can also be present together with K3K27me3 (a repressive mark) at bivalent genes poised for transcription. The exact role of the H3K4me3 SET domain of MLL in normal development is still unclear since mice expressing a

mutant MLL without the SET domain (MLLΔSET) are viable and fertile, although they displayed homeotic transformations and reduced hox expression [56].

Purification of the yeast set1 (COMPASS), mammalian MLL (mammalian MLL COMPASS-like) and *Drosophila* trx (*Drosophila* trithorax COMPASS-like) complexes revealed that many core components are indeed common between these complexes [57], further endorsing a conserved function of trx/set1/MLL in transcriptional regulation of target genes across different species. The core components of the drosophila trithorax COMPASS-like and mammalian MLL COMPASS-like complex (Fig. 11.3a) include WDR5, RBBP5, DPY30, ASH2L, which are similar to yeast COMPASS core components Cps30, Cps50, Cps25, Cps60 respectively. In contrast to the yeast COMPASS complex, *Drosophila* and mammalian COMPASS-like complexes contain MEN1 [57, 58], which binds to trx/MLL N-terminal sequences. There are also other critical cofactors not originally co-purified but later shown to interact with components of the mammalian COMPASS-like complex. These include (1) polymerase associated factor complex, PAFc, which is thought to play a

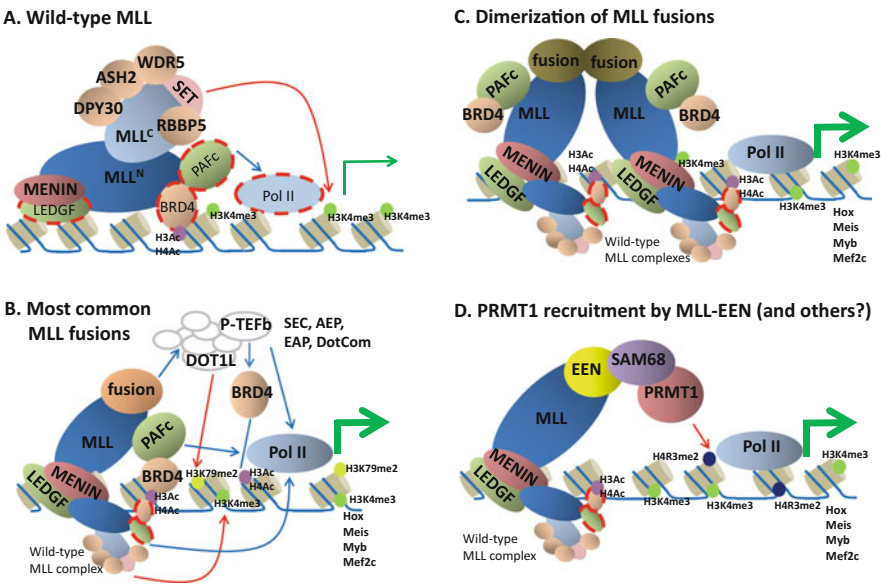

Fig.11.3 Schematic overview of the wild-type KMT2A/MLL complex and the different oncogenic transcriptional complexes formed by KMT2A/MLL fusions. (**a**) **Wild-type MLL complex**. Proteins not originally identified but shown to interact with components of the MLL COMPASS-like complex are marked with a red outline. PAF1 binds directly to MLL N-terminal sequences and interacts with BRD4. MEN1 interacts with LEDGF. RNA polymerase II is present at *MLL* target genes. (**b**–**d**) **Proposed different oncogenic transcriptional MLL fusion complexes**. *Green arrow* indicates transcription. *Blue arrows* indicate protein interactions. *Red arrows* indicate enzymatic histone methyltransferase activity. For clarity, no arrows have been depicted in the two panels on the right (**c** and **d**) except for PRMT1 enzymatic activity. Note that both wild-type MLL as well as MLL fusion proteins have been reported to co-localize at target gene promoters

role in targeting MLL complex to target loci [59]; (2) acetylated histone binding proteins of the BET family such as BRD4 potentially via interaction with PAFc [60]; and (3) the chromatin associated protein PSIP1/LEDGF via its interaction with MEN1 [61].

11.8.2 MLL Fusion Complexes

Over the past few years, it has become clear that multiple common MLL fusion partners co-exist in various endogenous complexes (EAP, AEP, SEC, DotCom) that are aberrantly recruited to MLL target genes resulting in abnormal transcriptional regulation of target genes such as *HOX* genes. These complexes often contain the H3K79me2 histone methyltransferase DOT1L and/or the positive elongation factor P-TEFb that is critical for the transition of transcriptional initiation to elongation by phosphorylating of the CTD of RNA polymerase II. Moreover MLL fusion complexes also interact with mammalian MLL COMPASS-like associated proteins such as MEN1, LEDGF and PAFc. Hence it is believed that these MLL fusions may transform haematopoietic cells by aberrant recruitment of histone modifying enzymes (e.g., DOT1L) and elongation factors (e.g., PAFc, P-TEFb) (Fig. 11.3b). For the less common MLL fusions, several alternative transformation mechanisms (Fig. 11.3c, d) have been described including (1) dimerization of MLL fusions observed in MLL-EPS15/AF1p and MLL-GAS7 [62], MLL-GPHN [63] and synthetic MLL-FKBP [14]; (2) recruitment of different histone modifying enzymes such as the protein-arginine methyltransferase, PRMT1 in MLL-SH3GL1/EEN [64]; or (3) indirect recruitment of AEP in case of MLL-MLLT4/AF6 [12], all resulting in de-regulated gene expression of critical targets such as *HOX* genes.

11.8.3 Crosstalk Between Wild-Type MLL and MLL Fusion Complexes

Interestingly, the N-terminus of MLL is preserved in all MLL fusions (Fig. 11.1), yet it is still unclear how wild-type and MLL fusion complexes are recruited to specific downstream targets. Proteins that interact within the N-terminus of MLL such as MEN1 and LEDGF are present in wild-type MLL as well as MLL fusion complexes suggesting putative binding to the same target genes. Indeed, up to 80 % of MLL-ENL target genes overlap with wild-type MLL targets [65]. On the other hand, MLL fusion target genes (n = 223) represent only a small minority (~7 %) of wild-type MLL targets (n = 2595), suggesting a highly selective binding exhibited by MLL-fusions to these loci. In contrast to wild-type MLL, all MLL fusions lack the C-terminal H3K4me3 SET domain. Yet, MLL fusion target genes are enriched not only with the H3K79me2 but also with the H3K4me3 chromatin mark [66, 67]

suggesting a possible crosstalk of MLL fusions and H3K4me3 HMTs. Although there are six different mammalian HMTs (MLL, KMT2B/MLL4, KMT2C/MLL3, KMT2D/MLL2, SETD1A and SETD1B) that can all contribute to the H3K4me3 mark, it is generally believed that MLL fusions may interact with wild-type MLL probably through proteins associated with both complexes. Supporting this hypothesis, experimental evidence for roles of PAFc and MEN1 in mediating this crosstalk has been reported. Wild-type MLL/PAFc complex is required for the efficient recruitment of MLL fusion to the same loci [68] as MLL fusion could not bind to *Hoxa9* in the absence of wild-type MLL or PAFc recruitment in MEFs. On the other hand, it was shown that knockout of *Men1* resulted in not only loss of H3K79me2 but also H3K4me3 at MLL fusion target genes in KMT2A/MLL fusion transduced cells [69], suggesting that enzymatic activities from wild-type MLL as well as MLL fusion complexes are acting in concert simultaneously on the *Hoxa9* gene locus. Consistently, wild-type MLL and MLL fusion complexes are recruited to *Hoxa9* in a Men1 dependent manner, and a conditional deletion of wild-type MLL inhibited MLL fusion mediated leukaemogenesis. While this suggests a critical role of wild-type MLL in *MLL* fusion mediated transformation, it should be noted that the conditional *MLL* mouse used in the Thiel et al. study suffered from a much more severe haematopoietic phenotype [70] than what has been reported for another conditional *MLL* mouse model [71]. It is not clear if these cells are generally compromised for any transformation. Interestingly, it was very recently reported that *MLL-AF9* AML is initiated and propagated normally in MLLΔSET haematopoietic cells [72] suggesting that the wild-type MLL H3K4me3 activity is dispensable for MLL fusion transformation. Furthermore, the leukaemic cell line ML2 [73], which was derived from an AML patient with *MLL-AF6*, lacks wild-type MLL. Further studies including comparison of both conditional mouse models and their impact on MLL fusion mediated transformation as well as the role of H3M4me3 and other histone modifications in MLL fusion mediated transformation will further advance our understanding of this fascinating crosstalk.

11.9 Pathways and Downstream Targets Critical in MLL Leukaemia

While characterizing the oncogenic MLL fusion transcriptional complexes in *MLL* leukaemia revealed several target genes and transcriptional programmes critical for self-renewal of *MLL* LSC [45, 46], recent data suggest that also other pathways and molecules which are neither under the direct control nor recruited to MLL fusion complexes play important roles in MLL leukaemia. Furthermore, it has been known for many years that several mutations such as *FLT3* and RAS genes recurrently found in AML and ALL patients also co-exist in MLL leukaemia patients, albeit their functional contribution, requirement and therapeutic value is much less clear. FLT3 has been reported to cooperate with some MLL fusions (e.g. MLL-ENL,

MLL-SEPT6) [74] and is expressed in high levels in *MLL* leukaemia patients [75, 76], but patient's responses in early FLT3 monotherapy clinical trials were rather limited both in depth and duration which severely dented the promise of FLT3 inhibition in acute leukaemia treatment [77]. Consistently, it was more recently shown that MLL fusions were able to induce leukaemia in *Flt3* knockout cells with expected latency and phenotype as by MLL fusion transformed wild type cells [78]. Moreover, co-expression of Flt3 or its constitutively activated mutant (Flt3-TKD) did not cooperate with MLL-AF4 in the transformation of human primary stem/progenitor cells [79] adding further doubt to the exact functional role and therapeutic value of Flt3 in *MLL* leukaemia.

11.9.1 MLL Downstream Targets

Identification of the oncogenic transcriptional MLL fusion complexes revealed that MLL fusions act as transcriptional activators and enhance gene expression of downstream targets. Among them, *Hox*, *Meis1*, *Pbx3*, *Myb* and *Mef2c* [30, 45, 80] represent the best characterized MLL downstream targets which are all part of a wider transcriptional programme critical for self-renewal of MLL LSCs. Knockdown or knockout of *Meis1*/*Pbx3* [81] or *Myb* [80] resulted in impaired MLL fusion cell growth and colony formation, whereas conditional deletion of *Mef2c* [82] did not impair induction or maintenance of MLL fusion mediated leukaemia but affected homing and invasiveness of MLL leukaemic cells in vivo. The functional role of *Hox* genes in MLL leukaemia remains controversial. While *HOXA9* has been shown to be required for human MLL leukaemia cell lines [83], which is in line with findings that *MLL-ENL* cannot transform *Hoxa9*−/− or *Hoxa7*−/− cells [84], it is also reported that both *MLL-AF9* and *MLL-GAS7* mediated transformations are independent of *Hoxa9* [85, 86]. Interestingly, only one microRNA, miR-495, is expressed at very low levels in *MLL* leukaemia compared to non-*MLL* leukaemia and it has been shown to target *Meis1* and *Pbx3* transcripts [87]. Indeed over-expression of miR-495 prolonged the latency of *MLL* leukaemia in vivo, providing a potential avenue to target the expression of certain MLL fusion downstream target genes.

More recently, MLL fusions have been reported to activate expression of the DNA demethylase *TET1* gene (ten eleven translocation 1) [88], and *miR-9* [89]. Moreover it has been shown that MLL fusions maintain the expression of *MECOM*/*EVI1* [90] in LSK (Lineage$^-$, Sca1$^+$, Kit$^+$) cells. However, expression of MLL fusion did not lead to an up-regulation of *MECOM*/*EVI1* in GMPs that have only basal expression levels of *MECOM*/*EVI1*. Common to these target genes is their requirement for MLL fusion mediated leukaemia as knockdown/depletion of *Tet1* and *Mecom*/*Evi1* has been reported to impair cell growth and leukaemia induction, whereas overexpression of *miR-9* promotes MLL-AF9 leukaemia in vivo.

11.9.2 Critical Pathways/Molecules Not Directly Regulated by MLL Fusions

11.9.2.1 Canonical WNT/beta-catenin

The evolutionarily conserved WNT/CTNNB1 pathway has been implicated in the pathogenesis of AML [91], and is required for the development of *MLL* LSCs [26, 92]. MLL fusions fail to induce leukaemia in the absence of CTNNB1 and its activation confers drug resistance to *MLL* LSCs [26]. Interestingly, deletion of *Ctnnb1* in haematopoietic cells has little/no effect on the function of adult haematopoiesis [93, 94], suggesting a therapeutic window. Consistently, pharmacological inhibition of Ctnnb1 by Indomethacin showed efficacy in inhibiting *MLL* leukaemia in vivo [95], although these experiments were done in serially transplanted animals probably due to the drug toxicity. It is noted that CTNNB1 also plays important roles in other haematological malignancies including CML-blast crisis [96–98], suggesting its broad therapeutic application.

11.9.2.2 ITGB3

In vivo shRNA screen carried out in a *MLL* mouse model identified integrin beta 3 (Itgb3) signalling amongst others as essential for MLL-AF9 mediated leukaemia [99]. Loss of Itgb3 signalling reversed the transcriptional programmes established by *MLL-AF9* such as self-renewal and led to up-regulation of differentiation programmes. Itgb3 dimerizes with Itgav on the cell surface, and transmits extra-cellular signals via a cascade of protein kinases such as Syk, Src and Ptk2b, guanine nucleotide exchange factors such as Vav1, Vav2, Vav3, and GTPases such as Rho and Cdc42. In the same study, Syk was identified and validated as a critical mediator of Itgb3 signalling in *MLL* leukaemia. Although the functional relationship between Itgb3/Syk and MLL fusion proteins in leukaemia are still largely unknown [100], the identification of tractable signalling molecules and the availability of Syk inhibitors that have already been shown effective in various diseases including B cell malignancies [101] add to our expanding repertoire of promising targets for *MLL* leukaemia.

11.9.2.3 Polycomb Group Proteins

Classically, trithorax and polycomb group proteins have been viewed as antagonistically acting proteins for regulating gene expression and cellular memory during normal development. However, increasing evidence suggests that polycomb proteins such as Bmi1 also play an important role in *MLL* leukaemia, which cannot be simply explained by their antagonistic functions [102, 103]. In addition, the polycomb protein CBX8, which has been shown to interact with the MLL fusion

partners ENL and AF9 [104], is critical for MLL-AF9 mediated transcriptional activation and transformation [105]. While it was suggested that CBX8 is critical for *MLL* leukaemia because of its recruitment of KAT5/TIP60 to the MLL fusion complex [105], another study provided evidence that the interaction between ENL and CBX8 allows MLL-ENL to inhibit the repressive function of polycomb group proteins on MLL fusion downstream target genes such as *HOX* [106]. Interestingly, two more polycomb group proteins (EZH2 and EED) genes have been reported to play key roles in *MLL* leukaemia [107, 108]. While the functional requirement of EZH2 in MLL leukaemia is less well defined, ablation of Eed impaired MLL-AF9 leukaemia although it is not clear if Eed is generally required for cell survival.

11.9.2.4 RUNX1

The heterodimeric core binding factors composed of RUNX1/AML1 and CBFB subunits are critical for definitive and adult normal haematopoiesis. Interestingly, RUNX1 and CBFB are also the most frequently translocated genes in acute leukaemia resulting in *RUNX1-RUNX1T1*/*AML1-ETO*/t(8;21) or *CBFB-MYH11*/inv(16) fusion genes. It has been recently shown that RUNX1 recruits wild-type MLL to activate RUNX1 target genes such as *SPI1*/*PU.1*, and the MLL-RUNX1 interaction prevents RUNX1 proteasome degradation [109]. Despite the RUNX1 interaction domain being located in the C-terminal portion of MLL, the N-terminus seemed required for enhancing RUNX1 protein levels, suggesting a differential effect of MLL fusion on RUNX1. Indeed a role for RUNX1 in MLL fusion mediated transformation was subsequently suggested since suppression of RUNX1 inhibited the growth of various MLL fusion transformed cells [110, 111]. However, it was very recently reported that MLL fusion down-regulates *RUNX1*/*CBFB* expression [112]. In contrast to the previous reports, down-regulation of *RUNX1*/*CBFB* expression levels accelerated MLL-AF9 leukaemia, whereas overexpression of *RUNX1* impaired *MLL-AF9* leukaemia. Therefore, further studies are needed to define the role and exact contribution of RUNX1 in *MLL* leukaemia.

11.9.2.5 Other Emerging Molecules

Involvement of myeloid specific transcription factors (such as CEBPA [113] and SPI1/PU.1 [114]), general transcription factor (NFKB [115]) and ubiquitin ligase (RNF20 [116]), which are all not under the direct transcriptional control of MLL fusion, further highlight a widespread crosstalk of other signalling pathways in *MLL* leukaemia. Genetic ablation/knockdown of *NFKB*, *Rnf20*, *Spi1*/*Pu.1*, or *Cebpa* impaired leukaemia growth. Interestingly, CEBPA is only important for the development of MLL fusion LSCs but not for their maintenance. While transcription factors are poor therapeutic targets to date, signalling cascades upstream of these

transcription factors may represent more promising targets. Inhibition of the IKK complex, a major upstream regulator of NFKB signalling suppresses cell growth and colony formation of murine *MLL* fusion transformed cells, suggesting that therapeutic targeting of this pathway is possible in *MLL* leukaemia. Moreover, a recently identified TNFa/NFKB autocrine positive feedback loop critical for the establishment of both MLL and non-MLL LSCs further highlights the role of NFKB, and the possibility of targeting TNFa for leukaemia therapy [117].

11.10 Current Therapies and Development of Novel Targeted Therapies

Currently, AML patients with 11q23 aberrations receive standard induction cytotoxic therapy "3 + 7" of daunorubicin and cytarabine, and they represent one of the worst prognostic subgroups [2]. Treatment of ALL patients, including those harbouring 11q23 abnormalities usually comprise a remission-induction phase, consolidation phase and continuation therapy [4]. While allogeneic HSCT is a key element in adult treatment, it confers no survival advantage in infant ALL with 11q23 aberrations and may only have limited benefits in a small subset of 11q23 patients, which possess additional poor prognostic factors [118, 119]. Strikingly, the drugs currently in use for these ALL and AML treatment regimens were developed in the 1950s–1970s. Although their dosage and schedule have been optimized resulting in higher survival rates accompanied by less general cytotoxicity in the majority of ALL cases and some AML cases, patients with 11q23 aberrations in general have had little to no benefits from these improvements, highlighting the need for novel drugs and therapies especially for this poor prognostic subgroup. The recent advances in identifying critical molecules, which are mainly part of the oncogenic MLL fusion complex and essential for *MLL* leukaemia, have provided novel targets for effective treatment, some of which are already being developed for early phases of clinical trials (Fig. 11.4).

11.10.1 Targets with Enzymatic Activities

11.10.1.1 DOT1L

DOT1L has been identified in several MLL fusion partner complexes (Fig. 11.2) and is essential for MLL fusion mediated leukaemia as demonstrated by conditional knockout of *Dot1l* for in vitro transformation [120] and in vivo leukaemogenesis [66, 122]. Noticeably, loss of Dot1l impaired haematopoiesis in all three different conditional knockout models, albeit with different severity possibly due to the use

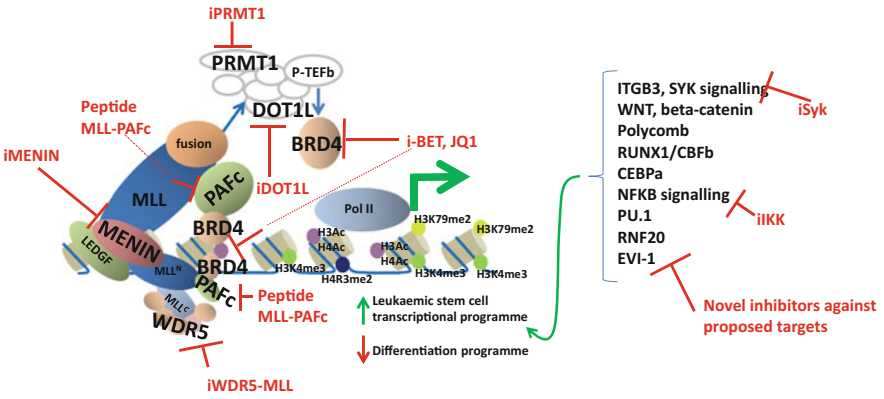

Fig. 11.4 Novel players in KMT2A/MLL leukaemia and development of targeted therapies. Schematic generic MLL fusion complex together with wild-type MLL complex at target gene locus driving distinct gene expression profiles. Highlighted in larger font size are individual molecules that have been shown important for MLL fusion-mediated transformation and have been experimentally targeted by small molecule inhibitors. On the right are emerging molecules and pathways listed with reported importance in MLL fusion-mediated transformation influencing MLL fusion protein transcriptional programmes. Small molecule inhibitors with already reported efficacy are indicated

of slightly different gene targeting strategies, Cre recombinases or/and incomplete Cre-mediated *Dot1l* deletion. Whereas one study found that loss of Dot1l had limited effects on haematopoietic progenitors [66], the others reported an indispensable function of Dot1l in the maintenance of adult haematopoiesis as the numbers and function of haematopoietic stem and progenitor cells were greatly compromised in the absence of Dot1l [120, 121]. Despite this controversy, the essential role of Dot1l in MLL leukaemia and its unique HMT enzymatic activity associated with non-SET domain makes Dot1l an attractive target. In fact, the first DOT1L inhibitor EPZ004777, a competitive analog of the co-factor S-adenosylmethionine (SAM), has been developed and shown to selectively kill *MLL* rearranged cells in vitro with reported IC50 levels 5–100× higher in non-*MLL* cells [123]. While EPZ004777 was tolerated in mice, it had quite poor in vivo pharmacokinetics, which only resulted in a very modest survival benefit in treated animals compared to the controls. The structural data on EPZ004777 binding to DOT1L revealed remodelling of the catalytic site of DOT1L, and identified positions of the compound that can be optimized to improve its pharmacokinetics and potency [124]. Indeed SGC0946, a brominated analogue of EPZ00477, has been reported to improve molecular effects on H3K79me2 level as well as selectively killing of *MLL* leukaemia cells. Another molecule evolved from EPZ004477 with improved potency is EPZ-5676 that has recently entered into phase I clinical trial [125]. Although future studies are needed to clarify their in vivo pharmacokinetic and efficacy on *MLL* leukaemia in the clinics, these studies demonstrate the promise of translating basic research results into potential patient's benefits.

11.10.1.2 PRMT1

Protein arginine methyltransferase 1 (PRMT1) with H4R3me2 specificity has been identified to be critical for MLL-EEN mediated transformation [64] as knockdown of PRMT1 resulted in impaired MLL-EEN in vitro transformation. Moreover, direct fusion of PRMT1 but not a catalytic dead mutant to the truncated MLL resulted in oncogenic transformation of primary murine haematopoietic stem/progenitor cells. While the role of *Prmt1* in other leukaemia remains to be determined, a small molecule inhibitor targeting PRMT1 has shown promising in vivo efficacy in a *MLL* fusion leukaemia model (N.C., B.B.Z. and C.W.E.S. unpublished data). Although EEN is a rare translocation partner of MLL, it is evident that transformation mediated by other MLL and non-MLL fusions may also depend on Prmt1 (N.C., B.B.Z. and C.W.E.S. unpublished data). Future studies using Prmt1 conditional knockout mouse and additional pharmacological PRMT1 inhibitors will be critical to clearly define the role of PRMT1 in *MLL* leukaemia and as a therapy target.

11.10.1.3 KDM1A/LSD1

Although LSD1 (KDM1A) mono- and di-demethylase does not directly associate with either wild-type MLL or MLL fusion complexes, shRNA mediated knockdown and pharmacological inhibition of LSD1 impaired in vitro growth and in vivo leukaemogenesis of cells transformed by various MLL fusions [126]. While inhibition of LSD1 induced differentiation of *MLL-AF9* transformed cells and spared normal bone marrow cells in vitro, its in vivo efficacy and the specificity (if any) towards MLL fusions remains unclear. Interestingly, another study reported that knockdown or pharmacological inhibition of LSD1 potentiated all-trans retinoic acid (ATRA) induced differentiation of non-*MLL* AML cells and reduced the engraftment ability of human primary AML samples in recipient mice [127]. These results suggest that inhibition of LSD1 alone or in combination with ATRA may have therapeutic value in treatment of other AMLs including those with 11q23 aberrations.

11.10.2 Targeting the Protein-Protein Interactions

11.10.2.1 BRD4

BRD4 is a member of the bromodomain containing proteins of the BET family that binds to acetylated histone, and may be recruited to MLL fusion complexes via its interaction with P-TEFb or PAF1. Its functional requirement in *MLL* leukaemia has recently been demonstrated in a shRNA screen [128]. Although BRD4 itself does not possess an enzymatic activity, small molecule inhibitors JQ1 [129] and i-BET [130] have been reported to competitively interfere with the binding of the

bromodomain to acetylated histones. In murine *MLL* fusion leukaemia models, treatment with JQ1 [128] or i-BET [60] has been very encouraging as it increased the survival of the treated animals compared to controls. Gene expression analysis showed that JQ1/i-BET treatment resulted in down-regulation of the LSC signature and reduced expression of *BCL2* and *MYC*. Transcriptional programmes masterminded by MYC have important roles in a variety of LSCs [131], suggesting that BRD4 inhibitors may have a broader efficacy and effects also in non *MLL* leukaemia. Indeed two recent reports highlighted the efficacy of i-BET in non-*MLL* AML [132] and in *JAK2*^{V617F} driven MPN [133]. They also observed down-regulation of *BCL2* and *MYC* upon treatment, suggesting that BET proteins control transcription of key targets such as MYC and BCL2, independently of the presence of MLL fusions proteins. Nevertheless, these studies provide a strong rationale to use BET inhibition as a novel experimental treatment for AML with and without 11q23 aberrations.

11.10.2.2 MEN1/MENIN

Another molecule associated with wild-type MLL as well as MLL fusion complexes is MEN1, which bridges MLL with the chromatin factor LEDGF. Interestingly, while MEN1 was originally identified as the product of the tumour suppressor gene *MEN1*, whose loss of function mutation causes sporadic neoplasm of various endocrine organs [134], it is critical for *MLL* fusion mediated transformation [61]. In its dual role as tumour suppressor and tumour promoting protein, structural analysis of free Men1 as well as Men1-MLL or Men1-JunD complexes revealed both MLL and JunD bind Men1 in the same pocket. However while Men1-JunD binding results in suppressing JunD-mediated transcription, Men1-MLL binding promotes MLL transcriptional activity [135]. Consequentially, MI-2, a small molecule inhibitor disrupting the Men1-MLL interaction was developed and showed in vitro efficacy in *MLL* fusion expressing cells [136]. Although the vivo efficacy has yet to be demonstrated, in vitro treatments of *MLL* fusion cells with MI-2 led to differentiation and down-regulation of MLL target genes with little effects on non-*MLL* transformed cells. Based on the high-resolution crystal structure of the Men1-MI-2 complex, a second generation inhibitor MI-2-2 has been developed and shows 7–9× higher affinity to Men1 compared to MI-2. Crucially, MI-2-2 displayed further enhanced cellular activities and more potent inhibition of MLL fusion harbouring human cell lines [137] although its in vivo efficacy has not been reported. It is noted that targeting the Men1-MLL interaction could potentially also amongst others inhibit the Men1-JunD interaction resulting in the conversion of proliferation suppressing JunD to proliferation promoting JunD with adverse consequences. Although targeting the Men1-MLL interaction may have therapeutic value in *MLL* leukaemia, Men1 is also a tissue specific tumour suppressor and small molecules targeting Men1-MLL or Men1 in general may have undesirable consequences in other tissues, which may result in the development of endocrine tumours. Future in vivo studies undoubtedly will shed lights onto these important issues.

11.10.2.3 WDR5

WDR5 is a component of the mammalian COMPASS-like MLL complex, which is not only absolutely required for its integrity but also for optimal MLL H3K4me3 activity [138]. Recently, a small molecule inhibitor MM-401 targeting the MLL1-WDR5 interaction has been reported and shows in vitro efficacy towards different MLL fusion proteins in primary cell line models and in patient samples [139]. Interestingly, while WDR5 is also found in other mammalian COMPASS and COMPASS-like complexes associated with different H3K4me3 methyltransferases (e.g., SETD1A, SETD1B, MLL, MLL2, MLL3, MLL4), MM-401 specifically inhibits the MLL1 COMPASS-like complex histone methyltransferase activity. Although its in vivo efficacy has yet to be demonstrated, the in vitro inhibition data provide further evidence that *MLL* fusion mediated transformation may be dependent on the presence of functional wild-type MLL COMPASS-like complex.

11.10.2.4 PAF1/PAFc

Polymerase associated factor complex (PAF1/PAFc) is recruited via MLL N-terminal sequences including its CXXC domain to both wild-type MLL and MLL fusion proteins. It has been reported that knockdown of CDC73 or CTR9, critical components of PAFc, inhibits MLL fusion transformation in vitro and that the interaction with PAF1 is necessary for MLL fusion transformation [59]. The use of a genetic model to delete *Cdc73* confirmed the initial findings, however it also affected non-*MLL* transformed cells raising questions about the specificity towards the MLL fusion [140]. Nevertheless, a competitive peptide that disrupts the binding of MLL fusion with PAF1 suppressed MLL fusion activity and did not adversely affect normal haematopoiesis [140]. These results suggest disruption of PAF1/MLL may also be a potential therapeutic avenue although its in vivo efficacy and potential translation into small molecule inhibitors need further investigations.

11.11 Concluding Remarks and Perspective

In the past decade, our understanding of the mechanisms mediating MLL fusion transformation of normal haematopoietic cells into leukaemic stem cells has dramatically expanded. Novel insights into the underlying molecular mechanisms and their crosstalk with other pathways/molecules have helped to identify a number of new therapeutic targets. Although development of small molecule inhibitors targeting these molecules with critical functions in *MLL* leukaemia is still in a very early stage, the successful application of DOT1L inhibitor, EPZ-5676 into phase I clinical trial for *MLL* leukaemia demonstrates the promise of translating our knowledge into novel treatments. Therefore continuous efforts in characterizing the molecular and

cellular basis of *MLL* leukaemia is expected to provide further important biological insights and more effective novel treatments for *MLL* leukaemia.

11.12 Appendix

During the production of this book chapter the first in vivo studies of MENIN inhibition in solid tumors and leukemia had been published [141, 142]. Using MI-2-2, a structure-based design combined with medical chemistry resulted in the development of MI-463 and MI-503 which improved survival of MLL leukaemic mice in vivo. Conversely, MI-463 or MI-503 had little impact on normal haematopoietic development. Although long-term experiments interrogating the effect of MI-463 and MI-503 on the MENIN-JunD interaction have not been performed, this work demonstrates the feasibility of targeting MENIN in MLL leukaemia.

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References

1. Tamai H, Inokuchi K (2010) 11q23/MLL acute leukemia: update of clinical aspects. J Clin Exp Hematopathol: JCEH 50(2):91–98
2. Zeisig BB et al (2012) SnapShot: acute myeloid leukemia. Cancer Cell 22(5):698–698, e1
3. Manola KN (2013) Cytogenetic abnormalities in acute leukaemia of ambiguous lineage: an overview. Br J Haematol 163:24–39
4. Pui C, Robison L, Look A (2008) Acute lymphoblastic leukaemia. Lancet 371:1030–1043
5. Greaves M, Wiemels J (2003) Origins of chromosome translocations in childhood leukaemia. Nat Rev Cancer 3(9):639–649
6. Hunger S, Sklar J, Link M (1992) Acute lymphoblastic leukemia occurring as a second malignant neoplasm in childhood: report of three cases and review of the literature. J Clin Oncol: Off J Am Soc Clin Oncol 10(1):156–163
7. Meyer C et al (2013) The MLL recombinome of acute leukemias in 2013. Leukemia 27(11):2165–2176
8. Zeisig DT et al (2005) The eleven-nineteen-leukemia protein ENL connects nuclear MLL fusion partners with chromatin. Oncogene 24(35):5525–5532
9. Bitoun E, Oliver P, Davies K (2007) The mixed-lineage leukemia fusion partner AF4 stimulates RNA polymerase II transcriptional elongation and mediates coordinated chromatin remodeling. Hum Mol Genet 16(1):92–106
10. Mueller D et al (2007) A role for the MLL fusion partner ENL in transcriptional elongation and chromatin modification. Blood 110(13):4445–4454
11. Lin C et al (2010) AFF4, a component of the ELL/P-TEFb elongation complex and a shared subunit of MLL chimeras, can link transcription elongation to leukemia. Mol Cell 37(3):429–437

12. Yokoyama A et al (2010) A higher-order complex containing AF4 and ENL family proteins with P-TEFb facilitates oncogenic and physiologic MLL-dependent transcription. Cancer Cell 17(2):198–212
13. Mohan M et al (2010) Linking H3K79 trimethylation to Wnt signaling through a novel Dot1-containing complex (DotCom). Genes Dev 24(6):574–589
14. Martin M et al (2003) Dimerization of MLL fusion proteins immortalizes hematopoietic cells. Cancer Cell 4(3):197–207
15. Bernot KM et al (2013) Eradicating acute myeloid leukemia in a Mll(PTD/wt):Flt3(ITD/wt) murine model: a path to novel therapeutic approaches for human disease. Blood 122(23):3778–3783
16. Zorko NA et al (2012) Mll partial tandem duplication and Flt3 internal tandem duplication in a double knock-in mouse recapitulates features of counterpart human acute myeloid leukemias. Blood 120(5):1130–1136
17. Zhang Y et al (2012) Stress hematopoiesis reveals abnormal control of self-renewal, lineage bias, and myeloid differentiation in Mll partial tandem duplication (Mll-PTD) hematopoietic stem/progenitor cells. Blood 120(5):1118–1129
18. Dohner K (2002) Prognostic significance of partial tandem duplications of the MLL gene in adult patients 16 to 60 years old with acute myeloid leukemia and normal cytogenetics: a study of the Acute Myeloid Leukemia Study Group Ulm. J Clin Oncol 20:3254–3261
19. Yip B, So C (2013) Mixed lineage leukemia protein in normal and leukemic stem cells. Exp Biol Med (Maywood) 238(3):315–323
20. Espinet B et al (2003) MLL intrachromosomal amplification in a pre-B acute lymphoblastic leukemia. Haematologica 88(2):28–29
21. Ziemin-van der Poel S et al (1991) Identification of a gene, MLL, that spans the breakpoint in 11q23 translocations associated with human leukemias. Proc Natl Acad Sci U S A 88(23):10735–10739
22. Gu Y et al (1992) The t(4;11) chromosome translocation of human acute leukemias fuses the ALL-1 gene, related to Drosophila trithorax, to the AF-4 gene. Cell 71(4):701–708
23. Djabali M et al (1992) A trithorax-like gene is interrupted by chromosome 11q23 translocations in acute leukaemias. Nat Genet 2(2):113–118
24. Corral J et al (1996) An Mll-AF9 fusion gene made by homologous recombination causes acute leukemia in chimeric mice: a method to create fusion oncogenes. Cell 85(6):853–861
25. Lavau C et al (1997) Immortalization and leukemic transformation of a myelomonocytic precursor by retrovirally transduced HRX-ENL. EMBO J 16(14):4226–4237
26. Yeung J et al (2010) β-Catenin mediates the establishment and drug resistance of MLL leukemic stem cells. Cancer Cell 18:606–618
27. Collins E et al (2000) Inter-chromosomal recombination of Mll and Af9 genes mediated by cre-loxP in mouse development. EMBO Rep 1(2):127–132
28. Drynan L et al (2005) Mll fusions generated by Cre-loxP-mediated de novo translocations can induce lineage reassignment in tumorigenesis. EMBO J 24(17):3136–3146
29. Forster A et al (2003) Engineering de novo reciprocal chromosomal translocations associated with Mll to replicate primary events of human cancer. Cancer Cell 3(5):449–458
30. Zeisig BB et al (2004) Hoxa9 and Meis1 are key targets for MLL-ENL-mediated cellular immortalization. Mol Cell Biol 24(2):617–628
31. Horton S et al (2005) Continuous MLL-ENL expression is necessary to establish a "Hox Code" and maintain immortalization of hematopoietic progenitor cells. Cancer Res 65(20):9245–9252
32. Wei J et al (2008) Microenvironment determines lineage fate in a human model of MLL-AF9 leukemia. Cancer Cell 13(6):483–495
33. Barabé F et al (2007) Modeling the initiation and progression of human acute leukemia in mice. Science (New York) 316(5824):600–604
34. So C et al (2003) MLL-GAS7 transforms multipotent hematopoietic progenitors and induces mixed lineage leukemias in mice. Cancer Cell 3(2):161–171

35. Chen W (2006) A murine Mll-AF4 knock-in model results in lymphoid and myeloid deregulation and hematologic malignancy. Blood 108:669–677
36. Metzler M et al (2006) A conditional model of MLL-AF4 B-cell tumourigenesis using invertor technology. Oncogene 25(22):3093–3103
37. Greaves MF, So CW (2006) Alert and sniffy-nosed about Mll-AF4? Blood 108:416–417
38. Krivtsov AV et al (2008) H3K79 methylation profiles define murine and human MLL-AF4 leukemias. Cancer Cell 14(5):355–368
39. Zeisig BB et al (2008) Reconstructing the disease model and epigenetic networks for MLL-AF4 leukemia. Cancer Cell 14(5):345–347
40. Bursen A et al (2010) The AF4.MLL fusion protein is capable of inducing ALL in mice without requirement of MLL.AF4. Blood 115(17):3570–3579
41. Kumar AR et al (2011) Authors' comments: t(4;11) leukemias display addiction to MLL-AF4 but not to AF4-MLL. Leuk Res 35(5):697
42. Kowarz E et al (2007) Complex MLL rearrangements in t(4;11) leukemia patients with absent AF4.MLL fusion allele. Leukemia 21(6):1232–1238
43. Zeisig BB et al (2003) The oncoprotein MLL-ENL disturbs hematopoietic lineage determination and transforms a biphenotypic lymphoid/myeloid cell. Oncogene 22(11):1629–1637
44. Cozzio A, Passegue E, Ayton PM, Karsunky H, Cleary ML, Weissman IL (2003) Similar MLL-associated leukemias arising from selfrenewing stem cells and short-lived myeloid progenitors. Genes Dev 17:3029–3035
45. Krivtsov AV et al (2006) Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. Nature 442(7104):818–822
46. Somervaille T et al (2009) Hierarchical maintenance of MLL myeloid leukemia stem cells employs a transcriptional program shared with embryonic rather than adult stem cells. Cell Stem Cell 4(2):129–140
47. Chen W et al (2008) Malignant transformation initiated by Mll-AF9: gene dosage and critical target cells. Cancer Cell 13(5):432–440
48. Urnov F et al (2005) Highly efficient endogenous human gene correction using designed zinc-finger nucleases. Nature 435(7042):646–651
49. Miller J et al (2011) A TALE nuclease architecture for efficient genome editing. Nat Biotechnol 29(2):143–148
50. Moehrle A, Paro R (1994) Spreading the silence: epigenetic transcriptional regulation during Drosophila development. Dev Genet 15:478–484
51. Yu B et al (1995) Altered hox expression and segmental identity in Mll-mutant mice. Nature 378(6556):505–508
52. Nislow C, Ray E, Pillus L (1997) SET1, a yeast member of the trithorax family, functions in transcriptional silencing and diverse cellular processes. Mol Biol Cell 8(12):2421–2436
53. Roguev A et al (2001) The Saccharomyces cerevisiae Set1 complex includes an Ash2 homologue and methylates histone 3 lysine 4. EMBO J 20(24):7137–7148
54. Milne T et al (2002) MLL targets SET domain methyltransferase activity to hox gene promoters. Mol Cell 10(5):1107–1117
55. Smith ST et al (2004) Modulation of heat shock gene expression by the TAC1 chromatin-modifying complex. Nat Cell Biol 6:162–167
56. Terranova R et al (2006) Histone and DNA methylation defects at hox genes in mice expressing a SET domain-truncated form of Mll. Proc Natl Acad Sci U S A 103(17):6629–6634
57. Mohan M et al (2010) Licensed to elongate: a molecular mechanism for MLL-based leukaemogenesis. Nat Rev Cancer 10(10):721–728
58. Mohan M et al (2011) The COMPASS family of H3K4 methylases in Drosophila. Mol Cell Biol 31(21):4310–4318
59. Muntean A et al (2010) The PAF complex synergizes with MLL fusion proteins at HOX loci to promote leukemogenesis. Cancer Cell 17(6):609–621
60. Dawson M et al (2011) Inhibition of BET recruitment to chromatin as an effective treatment for MLL-fusion leukaemia. Nature 478(7370):529–533

61. Yokoyama A et al (2005) The menin tumor suppressor protein is an essential oncogenic cofactor for MLL-associated leukemogenesis. Cell 123(2):207–218
62. So C et al (2003) Dimerization contributes to oncogenic activation of MLL chimeras in acute leukemias. Cancer Cell 4:99–110
63. Eguchi M, Eguchi-Ishimae M, Greaves M (2004) The small oligomerization domain of gephyrin converts MLL to an oncogene. Blood 103(10):3876–3882
64. Cheung N et al (2007) Protein arginine-methyltransferase-dependent oncogenesis. Nat Cell Biol 9(10):1208–1215
65. Wang Q-F et al (2011) MLL fusion proteins preferentially regulate a subset of wild-type MLL target genes in the leukemic genome. Blood 117(25):6895–6905
66. Bernt K et al (2011) MLL-rearranged leukemia is dependent on aberrant H3K79 methylation by DOT1L. Cancer Cell 20(1):66–78
67. Guenther M et al (2008) Aberrant chromatin at genes encoding stem cell regulators in human mixed-lineage leukemia. Genes Dev 22(24):3403–3408
68. Milne T et al (2010) Multiple interactions recruit MLL1 and MLL1 fusion proteins to the HOXA9 locus in leukemogenesis. Mol Cell 38(6):853–863
69. Thiel A et al (2010) MLL-AF9-induced leukemogenesis requires coexpression of the wild-type Mll allele. Cancer Cell 17(2):148–159
70. Jude C et al (2007) Unique and independent roles for MLL in adult hematopoietic stem cells and progenitors. Cell Stem Cell 1(3):324–337
71. McMahon K et al (2007) Mll has a critical role in fetal and adult hematopoietic stem cell self-renewal. Cell Stem Cell 1(3):338–345
72. Mishra BP et al (2014) The histone methyltransferase activity of MLL1 is dispensable for hematopoiesis and leukemogenesis. Cell Rep 7:1239–1247
73. Tanabe S et al (1996) Analysis of the t(6;11)(q27;q23) in leukemia shows a consistent breakpoint in AF6 in three patients and in the ML-2 cell line. Genes Chromosomes Cancer 15(4):206–216
74. Ono R et al (2005) Dimerization of MLL fusion proteins and FLT3 activation synergize to induce multiple-lineage leukemogenesis. J Clin Invest 115(4):919–929
75. Armstrong S et al (2002) MLL translocations specify a distinct gene expression profile that distinguishes a unique leukemia. Nat Genet 30(1):41–47
76. Armstrong S et al (2003) Inhibition of FLT3 in MLL. Validation of a therapeutic target identified by gene expression based classification. Cancer Cell 3(2):173–183
77. Knapper S (2011) The clinical development of FLT3 inhibitors in acute myeloid leukemia. Expert Opin Investig Drugs 20(10):1377–1395
78. Albouhair S, Morgado E, Lavau C (2013) Flt3 does not play a critical role in murine myeloid leukemias induced by MLL fusion genes. PLoS One 8(8):e72261
79. Montes R et al (2013) Ligand-independent FLT3 activation does not cooperate with MLL-AF4 to immortalize/transform cord blood CD34+ cells. Leukemia 28:666–674
80. Hess JL et al (2006) c-Myb is an essential downstream target for homeobox-mediated transformation of hematopoietic cells. Blood 108(1):297–304
81. Wong P et al (2007) Meis1 is an essential and rate-limiting regulator of MLL leukemia stem cell potential. Genes Dev 21(21):2762–2774
82. Schwieger M et al (2009) Homing and invasiveness of MLL/ENL leukemic cells is regulated by MEF2C. Blood 114(12):2476–2488
83. Faber J et al (2009) HOXA9 is required for survival in human MLL-rearranged acute leukemias. Blood 113(11):2375–2385
84. Ayton P, Cleary M (2003) Transformation of myeloid progenitors by MLL oncoproteins is dependent on Hoxa7 and Hoxa9. Genes Dev 17(18):2298–2307
85. So C et al (2004) Leukemic transformation of hematopoietic progenitors by MLL-GAS7 in the absence of Hoxa7 or Hoxa9. Blood 103(8):3192–3199
86. Kumar AR et al (2004) Hoxa9 influences the phenotype but not the incidence of Mll-AF9 fusion gene leukemia. Blood 103(5):1823–1828

87. Jiang X et al (2012) MiR-495 is a tumor-suppressor microRNA down-regulated in MLL-rearranged leukemia. Proc Natl Acad Sci U S A 109(47):19397–19402
88. Huang H et al (2013) TET1 plays an essential oncogenic role in MLL-rearranged leukemia. Proc Natl Acad Sci U S A 110(29):11994–11999
89. Chen P et al (2013) miR-9 is an essential oncogenic microRNA specifically overexpressed in mixed lineage leukemia-rearranged leukemia. Proc Natl Acad Sci U S A 110(28):11511–11516
90. Bindels E et al (2012) EVI1 is critical for the pathogenesis of a subset of MLL-AF9-rearranged AMLs. Blood 119(24):5838–5849
91. Mikesch JH et al (2007) The emerging role of Wnt signaling in the pathogenesis of acute myeloid leukemia. Leukemia 21:1638–1647
92. Wang Y et al (2010) The Wnt/β catenin pathway is required for the development of leukemia stem cells in AML. Science 327:1650–1653
93. Jeannet G et al (2008) Long-term, multilineage hematopoiesis occurs in the combined absence of beta-catenin and gamma-catenin. Blood 111(1):142–149
94. Koch U et al (2008) Simultaneous loss of beta- and gamma-catenin does not perturb hematopoiesis or lymphopoiesis. Blood 111(1):160–164
95. Fung T, Gandillet A, So C (2012) Selective treatment of mixed-lineage leukemia leukemic stem cells through targeting glycogen synthase kinase 3 and the canonical Wnt/β-catenin pathway. Curr Opin Hematol 19(4):280–286
96. Heidel F et al (2012) Genetic and pharmacologic inhibition of β-catenin targets imatinib-resistant leukemia stem cells in CML. Cell Stem Cell 10(4):412–424
97. Zhao C et al (2007) Loss of beta-catenin impairs the renewal of normal and CML stem cells in vivo. Cancer Cell 12(6):528–541
98. Jamieson CH et al (2004) Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. N Engl J Med 351(7):657–667
99. Miller P et al (2013) In Vivo RNAi screening identifies a leukemia-specific dependence on integrin beta 3 signaling. Cancer Cell 24(1):45–58
100. Zeisig BB, So CW (2013) Linking MLL leukemia with integrin signaling. Cancer Cell 24(1):5–7
101. Mócsai A, Ruland J, Tybulewicz V (2010) The SYK tyrosine kinase: a crucial player in diverse biological functions. Nat Rev Immunol 10(6):387–402
102. Zeisig BB et al (2011) Collaboration between PcG proteins and MLL fusions in leukemogenesis: an emerging paradigm. Cancer Cell 20(5):551–553
103. Smith LL et al (2011) Functional crosstalk between Bmi1 and MLL/Hoxa9 axis in establishment of normal hematopoietic and leukemic stem cells. Cell Stem Cell 8(6):649–662
104. García-Cuéllar M et al (2001) The ENL moiety of the childhood leukemia-associated MLL-ENL oncoprotein recruits human Polycomb 3. Oncogene 20(4):411–419
105. Tan J et al (2011) CBX8, a polycomb group protein, is essential for MLL-AF9-induced leukemogenesis. Cancer Cell 20(5):563–575
106. Maethner E et al (2013) MLL-ENL inhibits polycomb repressive complex 1 to achieve efficient transformation of hematopoietic cells. Cell Rep 3(5):1553–1566
107. Neff T et al (2012) Polycomb repressive complex 2 is required for MLL-AF9 leukemia. Proc Natl Acad Sci U S A 109(13):5028–5033
108. Tanaka S et al (2012) Ezh2 augments leukemogenicity by reinforcing differentiation blockage in acute myeloid leukemia. Blood 120(5):1107–1117
109. Huang G et al (2011) The ability of MLL to bind RUNX1 and methylate H3K4 at PU.1 regulatory regions is impaired by MDS/AML-associated RUNX1/AML1 mutations. Blood 118(25):6544–6552
110. Goyama S et al (2013) Transcription factor RUNX1 promotes survival of acute myeloid leukemia cells. J Clin Invest 123(9):3876–3888
111. Wilkinson A et al (2013) RUNX1 is a key target in t(4;11) leukemias that contributes to gene activation through an AF4-MLL complex interaction. Cell Rep 3(1):116–127

112. Zhao X et al (2014) Down-regulation of RUNX1/CBFβ by MLL fusion proteins enhances HSC self-renewal. Blood 123:1729–1738
113. Ohlsson E et al (2014) Initiation of MLL-rearranged AML is dependent on C/EBPα. J Exp Med 211(1):5–13
114. Zhou J et al (2013) PU.1 is essential for MLL leukemia partially via crosstalk with the MEIS/HOX pathway. Leukemia 28(7):1436–1438
115. Kuo H-P et al (2013) Epigenetic roles of MLL oncoproteins are dependent on NF-κB. Cancer Cell 24(4):423–437
116. Wang E et al (2013) Histone H2B ubiquitin ligase RNF20 is required for MLL-rearranged leukemia. Proc Natl Acad Sci U S A 110(10):3901–3906
117. Kagoya Y et al (2014) Positive feedback between NF-κB and TNF-α promotes leukemia-initiating cell capacity. J Clin Invest 124(2):528–542
118. Dreyer Z et al (2011) Analysis of the role of hematopoietic stem-cell transplantation in infants with acute lymphoblastic leukemia in first remission and MLL gene rearrangements: a report from the Children's Oncology Group. J Clin Oncol: Off J Am Soc Clin Oncol 29(2):214–222
119. Mann G et al (2010) Improved outcome with hematopoietic stem cell transplantation in a poor prognostic subgroup of infants with mixed-lineage-leukemia (MLL)-rearranged acute lymphoblastic leukemia: results from the Interfant-99 study. Blood 116(15):2644–2650
120. Jo S et al (2011) Requirement for Dot1l in murine postnatal hematopoiesis and leukemogenesis by MLL translocation. Blood 117(18):4759–4768
121. Nguyen A et al (2011) DOT1L, the H3K79 methyltransferase, is required for MLL-AF9-mediated leukemogenesis. Blood 117(25):6912–6922
122. Nguyen A et al (2011) Essential role of DOT1L in maintaining normal adult hematopoiesis. Cell Res 21(9):1370–1373
123. Daigle S et al (2011) Selective killing of mixed lineage leukemia cells by a potent small-molecule DOT1L inhibitor. Cancer Cell 20(1):53–65
124. Yu W et al (2012) Catalytic site remodelling of the DOT1L methyltransferase by selective inhibitors. Nat Commun 3:1288
125. Daigle S et al (2013) Potent inhibition of DOT1L as treatment of MLL-fusion leukemia. Blood 122(6):1017–1025
126. Harris W et al (2012) The histone demethylase KDM1A sustains the oncogenic potential of MLL-AF9 leukemia stem cells. Cancer Cell 21(4):473–487
127. Schenk T et al (2012) Inhibition of the LSD1 (KDM1A) demethylase reactivates the all-trans-retinoic acid differentiation pathway in acute myeloid leukemia. Nat Med 18(4):605–611
128. Zuber J et al (2011) RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia. Nature 478(7370):524–528
129. Filippakopoulos P et al (2010) Selective inhibition of BET bromodomains. Nature 468(7327):1067–1073
130. Nicodeme E et al (2010) Suppression of inflammation by a synthetic histone mimic. Nature 468(7327):1119–1123
131. Kim J et al (2010) A Myc network accounts for similarities between embryonic stem and cancer cell transcription programs. Cell 143(2):313–324
132. Dawson M et al (2014) Recurrent mutations, including NPM1c, activate a BRD4-dependent core transcriptional program in acute myeloid leukemia. Leukemia 28(2):311–320
133. Wyspiańska B et al (2014) BET protein inhibition shows efficacy against JAK2V617F-driven neoplasms. Leukemia 28(1):88–97
134. Chandrasekharappa S, Teh B (2003) Functional studies of the MEN1 gene. J Intern Med 253(6):606–615
135. Huang J et al (2012) The same pocket in menin binds both MLL and JUND but has opposite effects on transcription. Nature 482(7386):542–546
136. Grembecka J et al (2012) Menin-MLL inhibitors reverse oncogenic activity of MLL fusion proteins in leukemia. Nat Chem Biol 8(3):277–284

137. Shi A et al (2012) Structural insights into inhibition of the bivalent menin-MLL interaction by small molecules in leukemia. Blood 120(23):4461–4469
138. Dou Y et al (2006) Regulation of MLL1 H3K4 methyltransferase activity by its core components. Nat Struct Mol Biol 13(8):713–719
139. Cao F et al (2014) Targeting MLL1 H3K4 methyltransferase activity in mixed-lineage leukemia. Mol Cell 53(2):247–261
140. Muntean A et al (2013) MLL fusion protein-driven AML is selectively inhibited by targeted disruption of the MLL-PAFc interaction. Blood 122(11):1914–1922
141. Malik R et al (2015) Targeting the MLL complex in castration-resistant prostate cancer. Nat Med 21(4):344–352
142. Borkin D et al (2015) Pharmacologic inhibition of the Menin-MLL interaction blocks progression of MLL leukemia in vivo. Cancer Cell 27:589–602

Chapter 12
Acute Promyelocytic Leukaemia: From a Specific Translocation to Cure by Targeted Therapies

Kim L. Rice and Hugues de Thé

Contents

12.1 Introduction 252
12.2 APL: A Success Story of Targeted Therapy 254
12.2.1 APL Translocations: A Common Theme of Dysregulated Retinoid Signalling 254
12.2.2 The Changing Face of APL Pathology: Mechanisms Underlying PML-RARA-Induced APL 256
12.2.2.1 APL Mouse Models 257
12.2.2.2 Cooperating Events in Human APL 259
12.2.2.3 PML-RARA Effects on RXR Signalling 260
12.2.2.4 PML-RARA and Epigenetics 260
12.2.2.5 PML-RARA Control by Post Translational Modifications 261
12.2.2.6 Impact of PML-RARA on the Normal Function of PML 262
12.2.3 Novel Insights into APL Cure 263
12.2.3.1 RA-Induced Differentiation and Cure: Revisiting the Paradigm 263
12.2.3.2 Arsenic Frequently Cures APL 264
12.2.4 The APL Miracle: What Lessons Have We Learned? 266
References 266

Abstract Acute promyelocytic leukaemia (APL) biology started by the discovery of the driving t(15;17) translocation in 1977, followed by the unexpected ex vivo differentiation by a hormone, retinoic acid (RA), and the miraculous complete remissions that this drug yields in patients. This led to 25 years of extensive molecular explorations of the pathogenesis of this disease, starting with the identification

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of the retinoic acid receptor alpha (RARA) as the central target of all APL-associated translocations. The t(15;17) translocation associated with over 98 % of APL drives the expression of the PML-RARA fusion protein. The clinical activity of RA in a disease caused by an altered retinoic acid receptor constituted the first example of targeted therapy. How PML-RARA blocks differentiation and promotes self-renewal, but also how it confers RA-sensitivity, was the focus of intense investigations. While the first models emphasized the key role of transcriptional repression of RARA targets and subsequent reactivation by RA, further studies performed in animal models progressively lent weight to interference with PML function in transformation and PML-RARA degradation by RA in therapy response. The central role of PML-RARA degradation in therapy response was further supported by the discovery of the therapeutic activity of arsenic, a highly efficient APL drug, which also initiates PML-RARA degradation by targeting the PML moiety. Many studies investigated the pathogenesis of the most common variant t(11;17) translocations that yield a PLZF/RARA fusion. These rare APLs are distinctly much less sensitive to RA and completely resistant to arsenic. Finally, based on mouse models, clinical trials associating frontline RA and arsenic have demonstrated an extraordinary potency, definitively curing almost all cases of standard risk APL without any chemotherapy. Thus, through decades of basic research, cytogenetic analysis paved the way for the identification of the key PML-RARA driver, and molecular modelling of APL pathogenesis ultimately led to cure.

Keywords Retinoic acid • Arsenic • Proteolysis • SUMO • Targeted therapy

12.1 Introduction

Chromosomal translocations are primary events underlying the development of many cancers and have been identified in almost all tumour types, in addition to other non-malignant disorders such as schizophrenia and infertility [1]. The resulting rearrangement of genetic material between non-homologous chromosomes leads to the production of gene fusions which are estimated to be causal in ~20 % of human

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cancers [1]. Since the first molecular characterization of a translocation in 1982 by Croce and Leder, which led to the identification of the *MYC* oncogene in Burkitt lymphoma, numerous genes with critical roles in cell growth and haematopoiesis have been identified by studying translocation breakpoints [2, 3]. Subsequent functional analyses in animal models revealed that the products of these translocations are often drivers of disease, although the acquisition of secondary, cooperating mutations is likely necessary for full malignant transformation [4].

The clinical importance of translocations extends far beyond their role in disease initiation, since they are often used in the classification of specific disease subtypes, with major prognostic value. They are also used for the monitoring of residual disease upon therapy. The advent of the genomic era, which saw the complete sequencing of cancer genomes and global analyses of transcription factor binding, changes in the epigenetic landscape and gene expression patterns and copy number variation, has enabled unprecedented insights into the anatomy of the cancer genome. Such information has been essential for deciphering the consequences of gene translocations on a genome-wide level.

While recurrent chromosomal translocations have been identified across many tumour types, the great majority of them are associated with haematological malignancies. Indeed, 75 % of all translocations documented in human cancers pertain to haematological disorders [1]. In particular, translocations associated with acute myeloid leukaemia (AML) are well documented, with 267 recurrent balanced rearrangements, predominantly translocations, currently identified in AML, highlighting the significant influence of translocations in the aetiology of these leukaemias. It should be noted however, that while several AML subtypes are defined by one type of gene fusion, for example *BCR-ABL1* which is observed in near 100 % of chronic myelogenous leukaemia (CML) and *PML-RARA* which is expressed in ~98 % of acute promyelocytic leukaemia (APL), overall only ~20 % of AML are characterized by translocations or other balanced exchanges. The targets of these translocations include genes encoding tyrosine kinases (e.g. *ABL1*, *ALK*, *JAK2*) and nuclear transcription factors (e.g. *RUNX1*, *RARA*, *HOX11*), among others (e.g., *KMT2A/MLL*, *PML*, *NUP98*). Many of these are known to regulate critical cellular processes such as self-renewal, proliferation, apoptosis, senescence and differentiation, strengthening the idea that translocation products are key determinants of leukaemogenesis.

Two major outcomes of chromosomal translocations have been associated with malignancies. The first involves the activation of oncogenes by promoter insertion, typically due to aberrant rearrangements involving the T-cell receptor (TCR) or immunoglobulin (IG) loci, leading to the ectopic overexpression of a normal protein. The t(8;14)(q24.2;q32.3) translocation observed in the majority of Burkitt lymphoma is a classic example, whereby the *MYC* coding sequence is juxtaposed to the *IGH* enhancer. The second involves the production of chimeric fusion proteins with oncogenic properties, often due to modified gain of function, for example the PML-RARA fusion protein associated with the t(15;17) translocation in APL, as detailed below.

12.2 APL: A Success Story of Targeted Therapy

APL is a rare subtype of AML, whose story has gained significant attention in recent years, due to an unprecedented level of understanding of the physiopathology of the disease [5, 6] and also definitive cure rates that may be attributed to the use of two targeted therapies [7]. Since its association with the t(15;17)(q24.1;q21.1) translocation by Janet Rowley in 1977 [8], which was later shown to involve the *Promyelocytic Leukaemia* (*PML*) and *Retinoic Acid Receptor Alpha* (*RARA*) genes [9–11], APL has gone on to become one of the most well understood leukaemias, and a paradigm for personalized medicine. While treatment with chemotherapy led to remission in some patients, it was the introduction of retinoic acid (RA) and arsenic trioxide (ATO), two serendipitously discovered targeted agents, into standard of care regimes that progressively revolutionized the treatment of APL. Although the exquisite sensitivity of APL to these agents was discovered prior to our understanding of APL genetics and biology [12–14], the mechanisms underlying the therapeutic response to RA and ATO have now been extensively dissected, and they will likely have far reaching effects in the treatment of other malignancies.

12.2.1 APL Translocations: A Common Theme of Dysregulated Retinoid Signalling

APL is characterized by the aberrant self-renewal of cells blocked at the promyelocyte stage of differentiation. More than 98 % of patients harbour the t(15;17) translocation, generating the *PML-RARA* fusion transcript, giving rise to a protein containing a variable portion of PML, including the RING finger, B boxes and at least two coiled coil domains, joined to an invariant portion of RARA containing the DNA- and ligand-binding domains (Fig. 12.1).

Variability in the PML portion is dependent on the location of the breakpoints in the *PML* gene and alternative splicing, with most patients containing either the *PML-RARA* long (bcr1, intron 6) (~55 %) or short (bcr3, intron 3) forms (~40 %), whereas only a minority of patients contain the variable (bcr2, exon 6) form (~5 %) [15, 16]. *RARA* breakpoints all occur upstream of exon 4 (either in introns 1, 2 or 3), such that all fusion products contain RARA domains B-F [17]. In cases of APL arising after therapy, DNA breaks are likely mediated by topoisomerase II, activated by chemotherapeutic agents such as mitoxantrone. Therapy-linked APL is associated with a translocation breakpoint hotspot in intron 6 of *PML* [18]. In certain APL cases lacking the classical t(15;17) translocation, the *PML-RARA* fusion transcript may be generated by cryptic insertion events, whereby *RARA* is inserted into *PML* on 15q, or alternatively, *PML* is inserted into *RARA* on 17q [19]. Although reports vary, it appears that the incidence of cryptic rearrangements, deletion and complex variants leading to expression of *PML-RARA* may be as high as 14 % [20]. The identification of such cases highlight the utility of using reverse transcription-

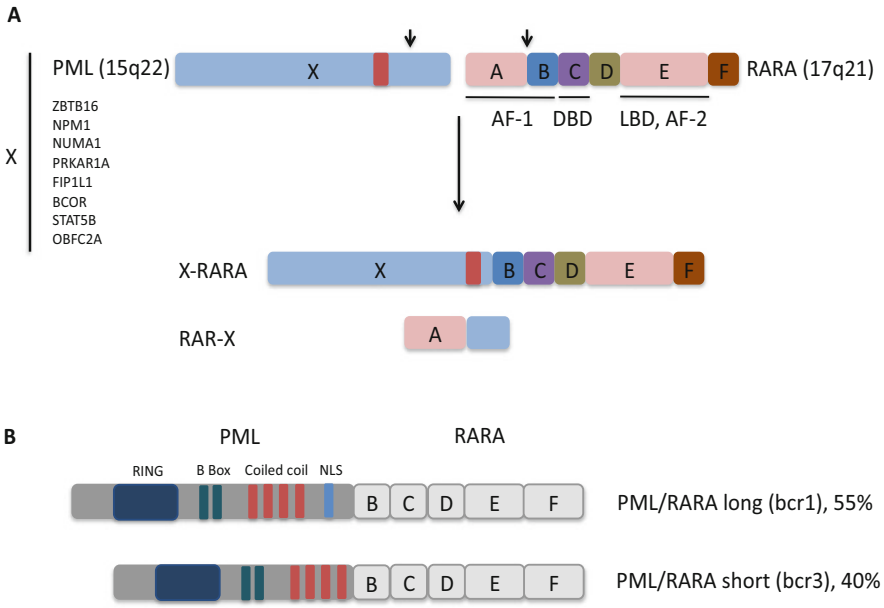

Fig. 12.1 (**a**) Chromosomal translocations in APL. Reciprocal chromosomal translocations lead to the generation of fusion proteins containing the amino terminal region of (*X*) proteins containing a self-association domain (*red box*) to the B-F domains of RARA, including the DNA and ligand binding domains. The reciprocal fusion protein containing the A domain of RARA may also be generated. Translocation breakpoints are indicated by *arrows*. (**b**) Major PML-RARA fusion proteins. PML-RARA fusions contain a variable portion of PML depending on the translocation breakpoint, leading to the production of PML-RARA long (bcr1), PML-RARA short (bcr3, intron 3) or PML-RARA variable (bcr2, exon 6) forms (not shown). All breakpoints in RARA occur upstream of exon 4 such that all fusion proteins contain RARA B-F domains. Notably, the PML-RARA short form does not contain the putative nuclear localization signal (*NLS*)

polymerase chain reaction (RT-PCR) to aid diagnosis, since *PML-RARA* transcripts may be present in patients in the absence of detectable cytogenetic changes.

The remaining 2 % of APL cases involve the fusion of RARA with other protein partners including ZBTB16 [21], NPM1 [22], NUMA1 [23], PRKAR1A [24], FIP1L1 [25], BCOR [26], STAT5B [27] and NABP1/OBFC2A [28], highlighting the role of dysregulated RARA and RA signalling in this specific subtype.

The importance of RA signalling in normal granulopoiesis is exemplified by studies of mice deprived of vitamin A [29] or treated with a pan RAR antagonist [30], who exhibit an expansion of the myeloid compartment in the bone marrow, spleen and peripheral blood. In vitamin A deficient mice, this is presumably the result of impaired apoptosis, and was reversible by addition of RA [29]. Although RARA is significantly expressed in granulocytes, *RARA* knockout mice do not appear to have defects in either the haematopoietic stem cell (HSC) compartment [31] or in normal granulopoiesis [30]. However, although RARA may be dispensable for normal granulopoiesis, RARA and its ligand, RA, clearly modulate differentiation

of this lineage, acting as a bidirectional regulator of granulopoiesis, limiting differentiation in the absence of RA and promoting differentiation in the presence of RA [30]. Based on this, it was postulated that APL fusion proteins may exert their oncogenic effects via enhancement of the normal inhibitory function of unliganded RARA receptors.

In non-APL cells, RARA binds DNA as a heterodimer with the retinoid X receptor (RXR) on to specific retinoic acid response elements (RAREs) present in the regulatory elements of target genes [32]. These elements are typically composed of two hexad half sites ((A/G)G(G/T)TCA) arranged in tandem repeats, separated by two to five nucleotides, although more complex variations on this theme have been observed [32–34]. In the unliganded state, RAR/RXR heterodimers interact with corepressors NCOR1 or NCOR2/SMRT, leading to recruitment of Sin3A and histone deacetylases (HDACs). The subsequent removal of acetyl groups from histones leads to condensation of chromatin and gene repression. Upon binding of ligand, however, interactions with these corepressors are destabilized, leading to their release and the recruitment of coactivators with intrinsic histone acetyltransferase (HAT) activity, including CREBBP, EP300, KAT2B/PCAF and SRC-1-3 [35, 36], culminating in gene activation.

Overexpression of PML-RARA in cell lines or primary mouse haematopoietic progenitors has pleiotropic effects that pertain to human APL, including inhibition of differentiation, block in apoptosis and increased self-renewal [37, 38]. In the classical scenario of APL pathogenesis, PML-RARA acts as a deviant retinoid receptor, forming oligomeric complexes via the coiled coil domain of PML, that are capable of enhanced recruitment of transcriptional corepressors and HDACs to RARA/RXR target promoters. In this model, the PML-RARA complex is rendered insensitive to physiological levels of RA due to the increase in corepressor stoichiometry, culminating in a block in myeloid differentiation that can only be released by treatment with pharmacological levels of RA. Yet, while this model recapitulated many features of RA response, it failed to explain the key role of arsenic in promoting APL cure [39].

12.2.2 The Changing Face of APL Pathology: Mechanisms Underlying PML-RARA-Induced APL

That PML-RARA drives transformation in t(15;17) APL is strongly supported by two lines of evidence. Firstly, the t(15;17) translocation represents the consistent genetic abnormality in the majority of APL, indicating that chimeric proteins produced by this translocation are key effectors of leukaemia initiation. Secondly, overexpression of PML-RARA in the myeloid compartment induces an APL-like disease in mice, albeit after a long latency [40–42]. However, the rather simplified model of APL pathogenesis described above has since evolved, and it is now clear that the oncogenic capacity of PML-RARA involves additional partner proteins, specific post-translational modifications and dysregulation of multiple pathways, particularly those involving PML (Fig. 12.2).

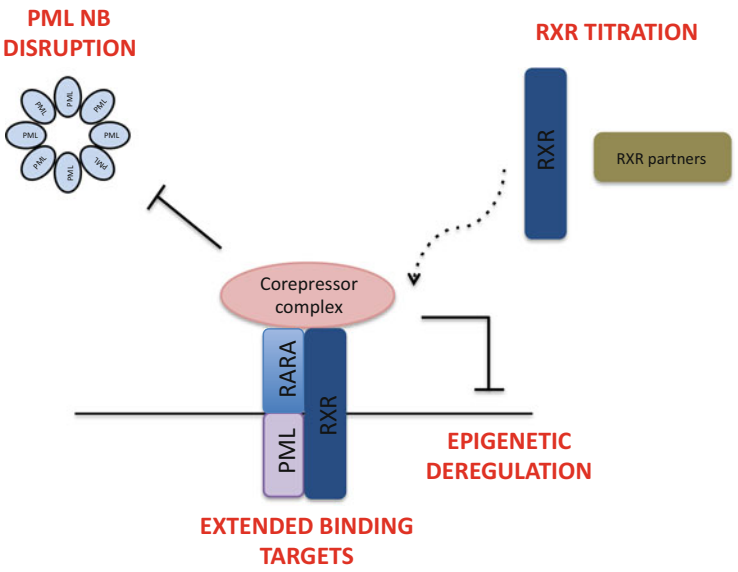

Fig. 12.2 Mechanisms of PML-RARA-induced APL. The PML-RARA fusion protein exerts multiple effects that may contribute to leukaemic transformation. PML-RARA may inhibit the functions of wild-type PML and disrupt PML nuclear bodies (NBs), which play important roles in mediating TP53 function, and have been associated with apoptosis, senescence and DNA-damage response. Functional PML-RARA complexes contain RXRA and exhibit a more relaxed DNA binding specificity when compared to wild-type RARA complexes, to include more widely spaced direct repeats. PML-RARA oligomers are capable of enhanced recruitment of co-repressors to target promoters, rendering these complexes insensitive to physiological concentrations of retinoic acid. PML-RARA may also recruit or affect the expression of chromatin modifying enzymes, leading to epigenetic deregulation of target genes. In addition to affecting RARA signalling, PML-RARA affects the thyroid receptor (TR), vitamin D3 receptor (VDR) and peroxisome proliferator activated receptor (PPARA) by titration of RXRA

12.2.2.1 APL Mouse Models

The evolution of murine models of leukaemia from retroviral bone marrow transduction and transplantation manipulations to more complex xeno-graft and transgenic models, has significantly enhanced our ability to understand how gene products from aberrant rearrangement events drive disease [43, 44].

Mouse models of APL have been particularly gratifying in this respect, yielding unique insights into the hierarchy of leukaemogenesis and treatment response. The first convincing evidence that PML-RARA drives transformation in t(15;17) APL came from transgenic mouse models demonstrating that overexpression of PML-RARA in the myeloid compartment under the hMRP8 [40] or cathespin G (CTSG) [41, 42] promoters, induced APL-like diseases. Although co-expression of the reciprocal RARA-PML fusion increased the penetrance of APL development in transgenic mice, expression alone did not alter myeloid development or yield APL, suggesting that it acts to potentiate PML-RARA-driven APL, but not as an

oncogene itself [45]. Indeed, the RARA-PML fusion is not consistently detected in patients harbouring the t(15;17) translocation [46], and in APL cases with cryptic *PML-RARA* rearrangements, *RARA-PML* transcripts were not detected [19]. Importantly, leukaemias derived from APL transgenic mice responded to treatment with RA, and this was associated with a disappearance of immature cells from the peripheral blood (PB) and restoration of normal PB parameters [47]. However, these mice relapsed soon after treatment was discontinued. Similarly, in the Kogan model, treatment of mice with RA was associated with loss of leukaemic cells (Gr-1^{low}/Mac-1^{low}) and the appearance of mature neutrophils in blood and bone marrow; however, survival was not increased [40]. Thus, while treatment of APL blasts is associated with differentiation, clear-cut evidence that RA-induced differentiation underlies the clinical response of patients to RA was lacking. For example, in the Ley model, treatment of mice with RA was associated with apoptosis rather than differentiation [41]. Subsequent analysis of these mice using various transplantation and treatment protocols revealed novel insights into the mechanisms underlying treatment response, to be discussed in detail below [38, 48, 49].

While these experiments provide undisputed evidence that PML-RARA drives disease initiation, APLs developed after a long latency, suggesting that additional changes (mutations, epigenetic modifications, post-translational modifications) acquired during the 'preleukaemic' phase are required for overt leukaemia. Indeed, in transgenic mice where PML-RARA expression was driven from the mouse *Pml* promoter in the context of Pml haploinsufficiency, APL was not observed, and instead, expression of PML-RARA led to an increase in the self-renewal capacity of a pool of haematopoietic progenitors. It was hypothesized that these cells may be more susceptible to acquiring secondary mutations, pushing them to the leukaemic phase [50]. Indeed, sequencing of an APL mouse genome identified a point mutation in *Jak1* (*Jak1*^{V657F}) which was identified in other APL mice, and deletion involving the histone demethylase, *Kdm6a* (*Utx*), which was also identified in other APL mice and a single case of human AML [51]. Thus, progression events are only partially shared between mice and humans, and this may reflect differences in the affinity or abundance of murine partners of PML-RARA as suggested by a recent study [52]. The distinct outcomes of these models may also reflect differences in the timing and/or level of PML-RARA expression, and provide an important basis for deconstructing the hierarchy of oncogenic events underlying APL.

Murine models have also been important for studying the more rare forms of APL, in particular that associated with t(11;17)(q23.2;q21.1) translocation, leading to the expression of the PLZF-RARA and reciprocal RARA-PLZF fusion proteins. In contrast to PML-RARA-driven APL, this subtype is less responsive to ATRA and patients have a poorer prognosis [53, 54]. Various hypotheses exist to explain why PLZF-RARA-driven APL is more refractory to treatment with pharmacologic levels of RA. Unlike PML-RARA, where the association with corepressors and HDACs is via the CoR box in the RARA moiety of the fusion, PLZF-RARA binds these factors via the POZ/BTB domain contained in the PLZF moiety, in addition to the CoR box, and this former association is associated with insensitivity to ATRA at high doses [55].

Whereas expression of PML-RARA alone from the *CTSG* promoter yielded an APL-like leukaemia in transgenic mice, overexpression of PLZF-RARA alone from the same promoter led to development of a more CML-like disease [47]. In keeping with the clinical observations, murine PLZF-RARA-driven APLs were more refractory to RA treatment compared to PML-RARA APL; however, some differentiation was still observed and survival was prolonged [47]. Interestingly, high dose RA treatment of PLZF/RARA APLs did lead to clinical remission, however, and this was attributed to the release of NCOR2 corepressor complexes [47]. The role of the reciprocal RARA-PLZF fusion was subsequently investigated. Generation of mice expressing both PLZF-RARA and RARA-PLZF led to a development of leukaemia with prominent APL features, including accumulation of immature promyelocytes in the BM [56]. Both single transgenic (ST) and double transgenic (DT) PLZF-RARA mice were significantly more refractory to RA treatment than PML-RARA-driven ones, however, interestingly, DT mice exhibited shorter survival after treatment, suggesting that the RARA-PLZF fusion protein modulates the response to RA [56]. In keeping with these observations, patients expressing both PLZF-RARA and RARA-PLZF were shown to be resistant to RA, whereas complete haematologic remission was observed in a patient expressing only PLZF-RARA [57].

12.2.2.2 Cooperating Events in Human APL

Although murine models of APL clearly demonstrated that the PML-RARA and PLZF-RARA fusion proteins are necessary for disease initiation, the preleukaemic phase observed in these mice suggests that at least some additional lesions are needed for malignant transformation. Analysis of human APL genomes using high-density single nucleotide polymorphism arrays revealed that ~40 % of patients have leukaemic cells with secondary abnormalities [58]. Among the potential candidates for cooperating secondary events identified by detailed analysis of murine and human APL genomes [51, 59, 60], *MYC* is particularly attractive, since even modest changes in *MYC* expression have been associated with significant phenotypic effects [61]. Indeed, complete or partial trisomy 8 is observed in ~12 % of human APL [59], and gain of *Myc* via trisomy for mouse chromosome 15 is observed in a significant number of transgenic APL mice (64 %) [59]. Furthermore, overexpression of *MYC* cooperated with PML-RARA in leukaemic transformation and suppressed gain of chromosome 15, highlighting a role for gain of chromosome 8 in APL [62].

Whole exome sequencing of APL patients also identified recurrent mutations in *FLT3*, *WT1*, *KRAS*, *CALR*, *CSMD1*, *DDR2*, *REV3L* and *TCERG1L*, with *FLT3* and *WT1* most often involved [60]. Mutations affecting *FLT3* are observed in 12–38 % (*FLT3*-ITD) and 2–20 % (*FLT3* TKD) of APL patients [63, 64], and mutant FLT3 has been shown to cooperate with PML-RARA in mouse APL models [65]. In addition to cooperating with PML-RARA for disease onset, aberrant FLT3 signalling may also impair response to therapy. Indeed, patients with a high *FLT3-ITD* burden

exhibited worse prognosis following treatment with RA and chemotherapy [66]. *KRAS* mutations are also observed in ~10 % of APL patients and coexpression with PML-RARA led to the development of highly penetrant APLs with short latency [67].

12.2.2.3 PML-RARA Effects on RXR Signalling

Unlike wild type RARA which requires RXR for efficient DNA binding, PML-RARA homodimers efficiently bind a variety of response elements in the absence of RXR [68], questioning the importance of RXR in APL development. However, accumulating evidence now indicates that RXRA plays a key role in PML-RARA-induced transformation. Firstly, PML-RARA oligomeric complexes have been shown to contain RXRA, and PML-RARA mutants defective for RXR binding were incapable of initiating APL in mice [69]. These in vitro observations were subsequently confirmed by genome-wide analysis of DNA binding by chromatin immuno-precipitation (ChIP) sequence analysis, which revealed near complete colocalization of PML-RARA and RXRA binding sites in APL cells [70]. Furthermore, RXR agonists are capable of activating transcription and initiating differentiation from PML-RARA responsive elements [71–73].

The PML-RARA/RXRA interaction has important biochemical consequences, since this complex has at least four DNA binding domains, thus affecting target specificity by allowing binding onto highly degenerate RAREs, representing a major gain of function [71]. Indeed, subsequent global ChIP analyses revealed that further to binding RAREs present in the promoters of RARA/RXR target genes, PML-RARA-complexes also bound, somewhat promiscuously, to various non-canonical RAREs, thus greatly extending the repertoire of genes regulated during normal retinoic acid signalling [70]. In addition to this novel gain of function, disruption of nuclear receptor signalling via titration of RXRs may also be a consequence of PML-RARA overexpression. PML-RARA was shown to sequester RXRA in cells [68], limiting the availability of RXRA required for other nuclear receptors. Indeed, RXRs enhance binding to several other nuclear receptors, in addition to RARA, to their specific response elements including the thyroid receptor (TR), vitamin D3 receptor (VDR) and peroxisome proliferator activated receptor (PPARA), highlighting the potentially far-reaching effects of PML-RARA overexpression on nuclear signalling.

12.2.2.4 PML-RARA and Epigenetics

The ability of PML-RARA complexes to induce epigenetic changes that contribute to malignant transformation is of particular interest, since unlike mutations, these marks are reversible, and may therefore be therapeutically targeted. Previous studies have shown that PML-RARA is capable of recruiting multiple chromatin modifying enzymes to the promoters of target genes, including HDACs [74], DNMTs [75] and polycomb repressive complexes (PRCs) [76], culminating in the repression

of specific gene promoters, and such activities have been linked to differentiation arrest and aberrant self-renewal. In the case of HDACs, genome-wide analysis revealed a correlation between ATRA-induced loss of PML-RARA/RXR binding, transcriptional activation and increased H3 acetylation [70]. Interestingly, similar observations were made upon RXRA excision [77]. Yet, evidence for a major modification on histone acetylation at PML-RARA binding sites in a non-overexpressing system is still lacking. Recent studies have further refined our understanding of the role of HDACs in the oncogenic complex. Intriguingly, in APL mice, Hdac1 was shown to have a dual role as an onco-suppressor in the pre-leukaemic phase, later playing an oncogenic role at the fully leukaemic stage [78]. This suggested that Hdac1 may have different functions within specific APL subpopulations and explained the ability of HDAC inhibitors such as valproic acid, to target the bulk population of APL cells while sparing leukaemia stem cells [79]. These findings clearly have implications for the clinical use of HDAC inhibitors, which have demonstrated some activity in APL [80].

The relationship between PML-RARA and DNA and histone methylation, is similarly unclear. While recruitment of DNMTs [75] and PRCs [76] to specific model promoters, such as the *RARb2* promoter, has been associated with changes in DNA and histone methylation, respectively, this relationship was not so evident on a global scale or in primary patient samples [81]. Indeed, genome-wide analyses of DNA and histone methylation in APL cells revealed only modest changes at PML-RARA binding sites in response to RA [70]. Treatment of APL cells with RA was associated with significant changes in K27 methylation, K9 methylation and DNA methylation, however only a minority of these differentially methylated regions overlapped with PML-RARA binding peaks [70] (Coline Gaillard, unpublished data). This could suggest that these changes are induced by differentiation and/or other chromatin modifying enzymes regulated by PML-RARA (such as JMJD3, SETDB1, JMJD1A and DNMT3A), as opposed to direct release of chromatin modifying enzymes from the PML-RARA/RXRA complex [70].

Another interesting outcome of global DNA binding analyses was the finding that a significant number of PML-RARA binding sites contained canonical SPI1/PU.1 motifs, many of which were in close proximity to RARE half sites [82]. PML-RARA repressed SPI1/PU.1-mediated transactivation of such sites, indicating that disruption of SPI1/PU.1-regulated genes by PML-RARA could constitute a major mechanism contributing to APL pathogenesis [82].

12.2.2.5 PML-RARA Control by Post Translational Modifications

Sumoylation of PML-RARA has also been shown to affect its transforming activity. Transcriptional repression by PML-RARA was dependent on sumoylation within the B1 box of PML, and mutation of this site abrogated recruitment of the death domain associated protein DAXX repressor, impairing leukaemia development in vivo [83]. Binding of PML-RARA to RXRA has also been shown to enhance RXRA sumoylation [69] and this likely contributes to transcriptional repression [77].

12.2.2.6 Impact of PML-RARA on the Normal Function of PML

In normal cells, PML is distributed throughout the nucleoplasm, but is also localized in distinct intra-nuclear domains, referred to as PML nuclear bodies (NBs) [84]. Immuno-histochemical analysis of normal bone marrow revealed that PML expression is predominantly restricted to myeloid cells, where distinct PML 'speckles' were observed throughout all stages of granulocytic differentiation [85]. In APL cells, these structures are disrupted, leading to a distinct micro-granular pattern that is reversed upon treatment with RA, suggesting that interference with normal PML function may contribute to pathogenesis [85–87]. This pattern was also normalized by arsenic, providing a major and unexpected convergence between the two drugs [88].

PML NBs are intra-nuclear domains that recruit numerous regulatory proteins (including TP53 and DAXX) and are associated with important processes, including DNA damage response, transcription, apoptosis and senescence [84]. These structures are assembled in response to oxidant stress [89], which may partially explain their association with sites of inflammation or transformation [90–92]. A central feature of many NB-associated proteins, including PML, is their modification by SUMO, which modulates protein function by affecting protein stability or stabilizing interactions [93]. In particular, the activity of TP53, a key regulator of cell senescence and apoptosis, is regulated by recruitment onto PML NBs, which are platforms for TP53 acetylation and activation [94]. PML may also function to precisely regulate TP53 activity by phosphorylation of CHEK2 in NBs [95] or by sequestering MDM2 to the nucleolus [96]. Recent studies showed that PML-induced senescence involved sequestration of RB1 and E2F proteins onto NBs, thereby inhibiting E2F transcription [97]. Mechanistically, this may occur by recruitment of E2F target promoters into close proximity to heterochromatin factors and HDACs in PML NBs.

Given the involvement of PML NBs in controlling key tumour suppressor processes, it is likely that disruption of NB-specific functions contributes to the oncogenic capacity of PML-RARA. Indeed, expression of PML-RARA in primary murine bone marrow allowed cells to bypass a senescence checkpoint and this was associated with disruption of a Pml NB-associated complex containing Daxx and Atrx [52]. Importantly, this was more pronounced when using a murine Pml within the PML-RARA fusion, suggesting that species-specific, Pml-dependent cellular interactions are key to the transformation process. Furthermore, studies comparing the potencies of different dimerization domains fused to RARA in initiating APL, demonstrated that only the PML coiled coil drives efficient transformation in vivo [98]. Similarly, the recent finding that a Pml-Tp53 checkpoint is required to eradicate leukaemia-initiating cells in a mouse model of APL [49] further strengthens the notion that interference of PML-RARA with the normal tumour suppressor functions of PML is key to both APL initiation, but also therapy (see below). However, whether disruption of PML NBs per se is absolutely required for APL remains unclear, particularly since these structures are not disrupted in PLZF-RARA-associated APL [19]. Other genetic alterations may compensate for this in this APL subset.

12.2.3 Novel Insights into APL Cure

12.2.3.1 RA-Induced Differentiation and Cure: Revisiting the Paradigm

Although the in vitro differentiating effects of ATRA on APL cells were observed in 1981 [99], the clinical benefit of ATRA was not realized until 1988, following the discovery that ATRA alone, and not synthetic agonists, could induce complete haematological remission in APL patients [12]. However, these patients eventually relapsed, and the detection of *PML-RARA* transcripts in blood from these patients [100], indicated that while ATRA alone could eliminate the bulk of leukaemic cells, an effect associated with differentiation of APL blasts, this was not sufficient to eliminate leukaemia initiating cells (LICs) capable of reinitiating disease.

A detailed investigation of the effect of RA on APL cell differentiation and clonogenicity ensued, and the results of these studies progressively challenged our view of APL as only a 'differentiation paradigm'. Indeed, in a murine model of t(15;17) APL, treatment of mice with increasing doses of RA induced morphologically similar levels of differentiation; however, loss of LICs (as assessed by the ability of treated APL cells to reinitiate APL in secondary recipients) was only observed at high doses of RA, indicating that the process of differentiation and cure could be, at least in part, uncoupled [38]. This distinction between differentiation and disease cure was also highlighted by the finding that APL blasts from PLZF-RARA DT mice (expressing PLZF-RARA and the reciprocal RARA-PLZF fusion) were sensitive to RA-induced differentiation, but that mice were almost completely resistant to treatment, even at high RA doses [38, 49]. Treatment of murine APLs with synthetic retinoids (acitretin and NRX195183) capable of activating PML-RARA-dependent transcription and inducing differentiation, but defective in their ability to degrade PML-RARA, also impaired LIC loss, highlighting the importance of PML-RARA degradation in disease clearance [101]. Subsequent analyses revealed that while both low and high RA doses induced differentiation of APL blasts, only high dose RA activated a senescence-like programme in leukaemic blasts and this was paralleled by PML-RARA degradation, Pml NB reformation and stabilization of p53 [49]. Indeed, loss of LIC activity was critically dependent on the presence of Pml and Tp53, directly implicating a Tp53-Pml axis in disease cure.

This model was recently substantiated in a resistant APL patient, following the identification of a mutation in the remaining allele of PML (A216V, adjacent to the arsenic binding site) that impedes arsenic-induced NB formation (Lehmann-Che, NEJM, in press). Truncated PML proteins incapable of forming NBs have also been identified in APL patients exhibiting RA resistance [102]. That treatment of patients with RA alone does not yield cure may reflect the fact that optimal concentrations of ATRA are not usually reached in patients. Notably, APL cure was observed in several patients treated intravenously with a liposomal form of ATRA, which may reflect higher intracellular concentrations of ATRA, paralleling these observations in mice [103]. Collectively these studies point to a model whereby degradation of PML-RARA and activation of PML and Tp53-dependent pathways are major factors underlying the success of RA therapy.

In the case of PLZF-RARA, the link between protein degradation and LIC clearance remains unclear. Indeed, similar to PML-RARA, PLZF-RARA is degraded following RA treatment [49, 104], however both patients and mice are refractory to ATRA treatment even at high doses of RA [49]. This resistance may be mediated by induction of genes by the reciprocal RARA-PLZF fusion, such as *CRABP1*, which plays a role in the catabolism of retinoids and has been associated with retinoic acid resistance [105, 106] or by PLZF-RARA, such as *MYC* [107]. PLZR-RARA has also been shown to recruit epigenetic machinery to target promoters [108], and this may lead to permanent silencing of genes, abrogating loss of LICS even following complete degradation of PLZF-RARA.

12.2.3.2 Arsenic Frequently Cures APL

Treatment of APL patients with arsenic trioxide as a single agent cures over 70 % of patients [39, 109]. Initial ex vivo studies in APL cells revealed a dose-dependent effect of arsenic, with high doses inducing apoptosis and low doses yielding partial differentiation [110], although differentiation becomes complete when cyclic-AMP [111] or CSF2/GM-CSF [112] are added. In vivo, treatment with arsenic was associated with differentiation and apoptosis, suggesting that the combination of these two processes may underlie the curative effect of arsenic on APL cells [39, 110].

Subsequent investigation into the mechanisms underlying this phenomenon revealed that arsenic, like RA, induces PML-RARA degradation, although via a biochemically distinct pathway [88]. At the molecular level, arsenic induces reactive oxygen species (ROS) that initiate the formation of PML intermolecular disulphide crosslinks, leading to PML oligomerization and NB formation [113]. Arsenic also binds cysteine residues in the RBCC domains of PML-RARA and PML, and this was proposed to facilitate UBE2I/UBC9 binding and subsequent sumoylation of PML [89, 114, 115]. PML hyper-sumoylation leads to the recruitment of RING finger protein 4 (RNF4), ultimately enforcing PML poly-ubiquitination and proteasome-mediated degradation [116, 117]. Importantly, these biochemical findings fit with the sequential wave of PML staining following arsenic treatment of primary APL cells or cell lines, starting with the reformation of PML NBs, the appearance of large PML staining aggregates and the later disappearance of these structures [88, 110]. The identification of arsenic resistant patients harbouring mutations in the B2 domain of PML-RARA that affect arsenic binding, sumoylation and degradation further highlights the link between PML-RARA degradation and APL cure [118, 119]. In the case of RA, PML-RARA degradation may occur either through the proteasome following ligand-dependent transcriptional activation [120], via a caspase 3-like activity targeting the PML portion of the PML-RARA fusion [121] or by autophagy [122]. Hence, the unexpected observation that both RA and arsenic target PML-RARA stability reinforces a model in which degradation of PML-RARA is the critical factor underlying APL cure [48, 123] (Fig. 12.3).

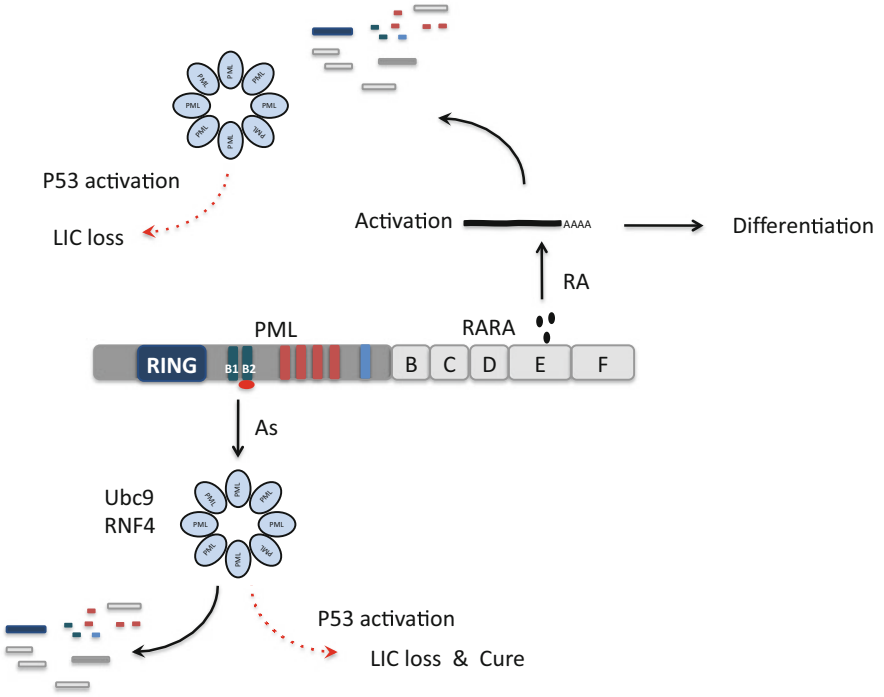

Fig. 12.3 Synergy of retinoic acid and arsenic for APL cure. Retinoic acid (*RA*) binds the ligand-binding domain within the RARA portion of PML-RARA, leading to release of co-repressor complexes, and transcriptional activation, accompanied by differentiation. PML-RARA is subsequently degraded, allowing the reformation of PML nuclear bodies (*NBs*) and activation of pathways culminating in leukaemia initiating cell (*LIC*) loss. Arsenic induces reactive oxygen species (*ROS*), enforcing PML and PML-RARA multimerization. Arsenic also binds to the B box 2 contained in the PML portion of PML-RARA, facilitating sumoylation by UBE2I/UBC9, ubiquitination by RNF4, targeting it for degradation by the proteasome

Comparison of the transcriptomic response of NB4 cells to RA and arsenic revealed that arsenic regulates significantly fewer genes compared to RA and the extent of regulation was also less profound [124]. Many genes were commonly regulated by both compounds suggesting that the differentiation enforced by As_2O_3 may be secondary to PML-RARA protein loss [39, 77]. Given that RA and As_2O_3 target PML-RARA degradation via distinct pathways, it was envisaged that these drugs would act synergistically to cure APL. Indeed a dramatic synergy was observed in murine models [125, 126] and was further substantiated by several clinical trials demonstrating that combined treatment of APL patients with RA and As_2O_3 leads to cure in virtually all patients, even in the absence of cytotoxic chemotherapy [7, 127].

12.2.4 The APL Miracle: What Lessons Have We Learned?

Malignancy is a multistep process that involves an initiating step, involving the dysregulation of a master transcriptional regulator afforded by recurrent balanced chromosomal translocations. That gene fusions arising from chromosomal translocations may represent an Achilles heel for cancers is substantiated by the sensitivity of certain subtypes of leukaemia, such as APL, to treatments targeting protein stability. Indeed, APL burden was significantly reduced in mouse APLs following targeted knockdown of the PML-RARA fusion protein, highlighting the dependency of leukaemic cells on continued PML-RARA expression [77] and further substantiating the role of therapy-induced degradation.

How applicable is the APL success story to other translocation-driven leukaemias? Is targeting oncoprotein degradation in other leukaemias a viable strategy? Can enforced PML NB reformation be harnessed to treat other malignancies? Some studies of adult T-cell leukaemia/lymphoma (ATL), an aggressive disease caused by infection with human T-lymphotropic virus type I (HTLV-I), and initiated by the viral regulatory protein, Tax [128] suggest that it might be the case. Indeed, combined IFN/arsenic treatment induces Tax degradation [129]. Importantly, the IFN/arsenic combination cures Tax-driven ATL in mice [130] or in newly diagnosed, chronic ATL patients [131], suggesting that targeted degradation of Tax may underlie therapy response. Moreover the biochemical mechanisms of Tax degradation appear largely similar to those of PML-RARA. Thus APL is not an exception, but the lessons that we have learnt pave the way to benefit the treatment of other diseases.

References

1. Mitelman F, Johansson B, Mertens F (2007) The impact of translocations and gene fusions on cancer causation. Nat Rev Cancer 7(4):233–245
2. Taub R, Kirsch I, Morton C, Lenoir G, Swan D et al (1982) Translocation of the c-myc gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells. Proc Natl Acad Sci U S A 79(24):7837–7841
3. Rabbitts TH (1994) Chromosomal translocations in human cancer. Nature 372(6502):143–149
4. Adams JM, Cory S (1991) Transgenic models of tumor development. Science 254(5035):1161–1167
5. de The H, Chen Z (2010) Acute promyelocytic leukaemia: novel insights into the mechanisms of cure. Nat Rev Cancer 10(11):775–783
6. Dos Santos GA, Kats L, Pandolfi PP (2013) Synergy against PML-RARa: targeting transcription, proteolysis, differentiation, and self-renewal in acute promyelocytic leukemia. J Exp Med 210(13):2793–2802
7. Lo-Coco F, Avvisati G, Vignetti M, Thiede C, Orlando SM et al (2013) Retinoic acid and arsenic trioxide for acute promyelocytic leukemia. N Engl J Med 369(2):111–121
8. Rowley JD, Golomb HM, Dougherty C (1977) 15/17 translocation, a consistent chromosomal change in acute promyelocytic leukaemia. Lancet 1(8010):549–550
9. de The H, Chomienne C, Lanotte M, Degos L, Dejean A (1990) The t(15;17) translocation of acute promyelocytic leukaemia fuses the retinoic acid receptor alpha gene to a novel transcribed locus. Nature 347(6293):558–561

10. Borrow J, Goddard AD, Sheer D, Solomon E (1990) Molecular analysis of acute promyelocytic leukemia breakpoint cluster region on chromosome 17. Science 249(4976):1577–1580
11. de The H, Lavau C, Marchio A, Chomienne C, Degos L et al (1991) The PML-RAR alpha fusion mRNA generated by the t(15;17) translocation in acute promyelocytic leukemia encodes a functionally altered RAR. Cell 66(4):675–684
12. Huang ME, Ye YC, Chen SR, Chai JR, Lu JX et al (1988) Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia. Blood 72(2):567–572
13. Sun H, Ma L, Hu X, Zhang T (1992) Chin J Integr Chin Tradit Med West Med 12:170–171
14. Zhang P, Wang S, Hu X (1996) Chin J Hematol 17:58–60
15. Pandolfi PP, Alcalay M, Fagioli M, Zangrilli D, Mencarelli A et al (1992) Genomic variability and alternative splicing generate multiple PML/RAR alpha transcripts that encode aberrant PML proteins and PML/RAR alpha isoforms in acute promyelocytic leukaemia. EMBO J 11(4):1397–1407
16. Dong S, Geng JP, Tong JH, Wu Y, Cai JR et al (1993) Breakpoint clusters of the PML gene in acute promyelocytic leukemia: primary structure of the reciprocal products of the PML-RARA gene in a patient with t(15;17). Genes Chromosomes Cancer 6(3):133–139
17. Zelent A, Guidez F, Melnick A, Waxman S, Licht JD (2001) Translocations of the RARalpha gene in acute promyelocytic leukemia. Oncogene 20(49):7186–7203
18. Mistry AR, Felix CA, Whitmarsh RJ, Mason A, Reiter A et al (2005) DNA topoisomerase II in therapy-related acute promyelocytic leukemia. N Engl J Med 352(15):1529–1538
19. Grimwade D, Gorman P, Duprez E, Howe K, Langabeer S et al (1997) Characterization of cryptic rearrangements and variant translocations in acute promyelocytic leukemia. Blood 90(12):4876–4885
20. Amare PK, Baisane C, Nair R, Menon H, Banavali S et al (2011) Characterization of cryptic rearrangements, deletion, complex variants of PML, RARA in acute promyelocytic leukemia. Indian J Hum Genet 17(2):54–58
21. Chen Z, Brand NJ, Chen A, Chen SJ, Tong JH et al (1993) Fusion between a novel Kruppel-like zinc finger gene and the retinoic acid receptor-alpha locus due to a variant t(11;17) translocation associated with acute promyelocytic leukaemia. EMBO J 12(3):1161–1167
22. Redner RL, Rush EA, Faas S, Rudert WA, Corey SJ (1996) The t(5;17) variant of acute promyelocytic leukemia expresses a nucleophosmin-retinoic acid receptor fusion. Blood 87(3):882–886
23. Wells RA, Catzavelos C, Kamel-Reid S (1997) Fusion of retinoic acid receptor alpha to NuMA, the nuclear mitotic apparatus protein, by a variant translocation in acute promyelocytic leukaemia. Nat Genet 17(1):109–113
24. Catalano A, Dawson MA, Somana K, Opat S, Schwarer A et al (2007) The PRKAR1A gene is fused to RARA in a new variant acute promyelocytic leukemia. Blood 110(12):4073–4076
25. Kondo T, Mori A, Darmanin S, Hashino S, Tanaka J et al (2008) The seventh pathogenic fusion gene FIP1L1-RARA was isolated from a t(4;17)-positive acute promyelocytic leukemia. Haematologica 93(9):1414–1416
26. Yamamoto Y, Tsuzuki S, Tsuzuki M, Handa K, Inaguma Y et al (2010) BCOR as a novel fusion partner of retinoic acid receptor alpha in a t(X;17)(p11;q12) variant of acute promyelocytic leukemia. Blood 116(20):4274–4283
27. Arnould C, Philippe C, Bourdon V, Gr goire MJ, Berger R et al (1999) The signal transducer and activator of transcription STAT5b gene is a new partner of retinoic acid receptor alpha in acute promyelocytic-like leukaemia. Hum Mol Genet 8(9):1741–1749
28. Won D, Shin SY, Park CJ, Jang S, Chi HS et al (2013) OBFC2A/RARA: a novel fusion gene in variant acute promyelocytic leukemia. Blood 121(8):1432–1435
29. Kuwata T, Wang IM, Tamura T, Ponnamperuma RM, Levine R et al (2000) Vitamin A deficiency in mice causes a systemic expansion of myeloid cells. Blood 95(11):3349–3356
30. Kastner P, Lawrence HJ, Waltzinger C, Ghyselinck NB, Chambon P et al (2001) Positive and negative regulation of granulopoiesis by endogenous RARalpha. Blood 97(5):1314–1320

31. Purton LE, Dworkin S, Olsen GH, Walkley CR, Fabb SA et al (2006) RARgamma is critical for maintaining a balance between hematopoietic stem cell self-renewal and differentiation. J Exp Med 203(5):1283–1293
32. Chambon P (1996) A decade of molecular biology of retinoic acid receptors. FASEB J 10(9):940–954
33. de The H, Vivanco-Ruiz MM, Tiollais P, Stunnenberg H, Dejean A (1990) Identification of a retinoic acid responsive element in the retinoic acid receptor beta gene. Nature 343(6254):177–180
34. Mangelsdorf DJ, Evans RM (1995) The RXR heterodimers and orphan receptors. Cell 83(6):841–850
35. Onate SA, Tsai SY, Tsai MJ, O'Malley BW (1995) Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 270(5240):1354–1357
36. Yao TP, Ku G, Zhou N, Scully R, Livingston DM (1996) The nuclear hormone receptor coactivator SRC-1 is a specific target of p300. Proc Natl Acad Sci U S A 93(20):10626–10631
37. Grignani F, Ferrucci PF, Testa U, Talamo G, Fagioli M et al (1993) The acute promyelocytic leukemia-specific PML-RAR alpha fusion protein inhibits differentiation and promotes survival of myeloid precursor cells. Cell 74(3):423–431
38. Nasr R, Guillemin MC, Ferhi O, Soilihi H, Peres L et al (2008) Eradication of acute promyelocytic leukemia-initiating cells through PML-RARA degradation. Nat Med 14(12):1333–1342
39. Zhu J, Chen Z, Lallemand-Breitenbach V, de The H (2002) How acute promyelocytic leukaemia revived arsenic. Nat Rev Cancer 2(9):705–713
40. Brown D, Kogan S, Lagasse E, Weissman I, Alcalay M et al (1997) A PMLRARalpha transgene initiates murine acute promyelocytic leukemia. Proc Natl Acad Sci U S A 94(6):2551–2556
41. Grisolano JL, Wesselschmidt RL, Pelicci PG, Ley TJ (1997) Altered myeloid development and acute leukemia in transgenic mice expressing PML-RAR alpha under control of cathepsin G regulatory sequences. Blood 89(2):376–387
42. He LZ, Tribioli C, Rivi R, Peruzzi D, Pelicci PG et al (1997) Acute leukemia with promyelocytic features in PML/RARAlpha transgenic mice. Proc Natl Acad Sci U S A 94(10):5302–5307
43. Piazza F, Gurrieri C, Pandolfi PP (2001) The theory of APL. Oncogene 20(49):7216–7222
44. Ablain J, Nasr R, Zhu J, Bazarbachi A, Lallemand-Breittenbach V et al (2013) How animal models of leukaemias have already benefited patients. Mol Oncol 7(2):224–231
45. Pollock JL, Westervelt P, Kurichety AK, Pelicci PG, Grisolano JL et al (1999) A bcr-3 isoform of RARalpha-PML potentiates the development of PML-RARalpha-driven acute promyelocytic leukemia. Proc Natl Acad Sci U S A 96(26):15103–15108
46. Grimwade D, Howe K, Langabeer S, Davies L, Oliver F et al (1996) Establishing the presence of the t(15;17) in suspected acute promyelocytic leukaemia: cytogenetic, molecular and PML immunofluorescence assessment of patients entered into the M.R.C. ATRA trial. M.R.C. Adult Leukaemia Working Party. Br J Haematol 94(3):557–573
47. He LZ, Guidez F, Tribioli C, Peruzzi D, Ruthardt M et al (1998) Distinct interactions of PML-RARalpha and PLZF-RARalpha with co-repressors determine differential responses to RA in APL. Nat Genet 18(2):126–135
48. Lallemand-Breitenbach V, Zhu J, Chen Z, de The H (2012) Curing APL through PML/RARA degradation by As2O3. Trends Mol Med 18(1):36–42
49. Ablain J, Rice K, Soilihi H, de Reynies A, Minucci S et al (2014) Activation of a promyelocytic leukemia-tumor protein 53 axis underlies acute promyelocytic leukemia cure. Nat Med 20(2):167–174
50. Welch JS, Yuan W, Ley TJ (2011) PML-RARA can increase hematopoietic self-renewal without causing a myeloproliferative disease in mice. J Clin Invest 121(4):1636–1645

51. Wartman LD, Larson DE, Xiang Z, Ding L, Chen K et al (2011) Sequencing a mouse acute promyelocytic leukemia genome reveals genetic events relevant for disease progression. J Clin Invest 121(4):1445–1455
52. Korf K, Wodrich H, Haschke A, Ocampo C, Harder L et al (2014) The PML domain of PML-RARalpha blocks senescence to promote leukemia. Proc Natl Acad Sci U S A 111(33):12133–12138
53. Guidez F, Huang W, Tong JH, Dubois C, Balitrand N et al (1994) Poor response to all-trans retinoic acid therapy in a t(11;17) PLZF/RAR alpha patient. Leukemia 8(2):312–317
54. Licht JD, Chomienne C, Goy A, Chen A, Scott AA et al (1995) Clinical and molecular characterization of a rare syndrome of acute promyelocytic leukemia associated with translocation (11;17). Blood 85(4):1083–1094
55. Grignani F, De Matteis S, Nervi C, Tomassoni L, Gelmetti V et al (1998) Fusion proteins of the retinoic acid receptor-alpha recruit histone deacetylase in promyelocytic leukaemia. Nature 391(6669):815–818
56. He LZ, Bhaumik M, Tribioli C, Rego EM, Ivins S et al (2000) Two critical hits for promyelocytic leukemia. Mol Cell 6(5):1131–1141
57. Petti MC, Fazi F, Gentile M, Diverio D, De Fabritiis P et al (2002) Complete remission through blast cell differentiation in PLZF/RARalpha-positive acute promyelocytic leukemia: in vitro and in vivo studies. Blood 100(3):1065–1067
58. Akagi T, Shih LY, Kato M, Kawamata N, Yamamoto G et al (2009) Hidden abnormalities and novel classification of t(15;17) acute promyelocytic leukemia (APL) based on genomic alterations. Blood 113(8):1741–1748
59. Le Beau MM, Bitts S, Davis EM, Kogan SC (2002) Recurring chromosomal abnormalities in leukemia in PML-RARA transgenic mice parallel human acute promyelocytic leukemia. Blood 99(8):2985–2991
60. Riva L, Ronchini C, Bodini M, Lo-Coco F, Lavorgna S et al (2013) Acute promyelocytic leukemias share cooperative mutations with other myeloid-leukemia subgroups. Blood Cancer J 3:e147
61. Reavie L, Buckley SM, Loizou E, Takeishi S, Aranda-Orgilles B et al (2013) Regulation of c-Myc ubiquitination controls chronic myelogenous leukemia initiation and progression. Cancer Cell 23(3):362–375
62. Jones L, Wei G, Sevcikova S, Phan V, Jain S et al (2010) Gain of MYC underlies recurrent trisomy of the MYC chromosome in acute promyelocytic leukemia. J Exp Med 207(12):2581–2594
63. Beitinjaneh A, Jang S, Roukoz H, Majhail NS (2010) Prognostic significance of FLT3 internal tandem duplication and tyrosine kinase domain mutations in acute promyelocytic leukemia: a systematic review. Leuk Res 34(7):831–836
64. Noguera NI, Breccia M, Divona M, Diverio D, Costa V et al (2002) Alterations of the FLT3 gene in acute promyelocytic leukemia: association with diagnostic characteristics and analysis of clinical outcome in patients treated with the Italian AIDA protocol. Leukemia 16(11):2185–2189
65. Kelly LM, Kutok JL, Williams IR, Boulton CL, Amaral SM et al (2002) PML/RARAlpha and FLT3-ITD induce an APL-like disease in a mouse model. Proc Natl Acad Sci U S A 99(12):8283–8288
66. Schnittger S, Bacher U, Haferlach C, Kern W, Alpermann T et al (2011) Clinical impact of FLT3 mutation load in acute promyelocytic leukemia with t(15;17)/PML-RARA. Haematologica 96(12):1799–1807
67. Chan IT, Kutok JL, Williams IR, Cohen S, Moore S et al (2006) Oncogenic K-ras cooperates with PML-RAR alpha to induce an acute promyelocytic leukemia-like disease. Blood 108(5):1708–1715
68. Perez A, Kastner P, Sethi S, Lutz Y, Reibel C et al (1993) PMLRAR homodimers: distinct DNA binding properties and heteromeric interactions with RXR. EMBO J 12(8):3171–3182

69. Zhu J, Nasr R, Peres L, Riaucoux-Lormiere F, Honore N et al (2007) RXR is an essential component of the oncogenic PML/RARA complex in vivo. Cancer Cell 12(1):23–35
70. Martens JH, Brinkman AB, Simmer F, Francoijs KJ, Nebbioso A et al (2010) PML-RARalpha/RXR alters the epigenetic landscape in acute promyelocytic leukemia. Cancer Cell 17(2):173–185
71. Kamashev D, Vitoux D, De The H (2004) PML-RARA-RXR oligomers mediate retinoid and rexinoid/cAMP cross-talk in acute promyelocytic leukemia cell differentiation. J Exp Med 199(8):1163–1174
72. Altucci L, Rossin A, Hirsch O, Nebbioso A, Vitoux D et al (2005) Rexinoid-triggered differentiation and tumor-selective apoptosis of acute myeloid leukemia by protein kinase A-mediated desubordination of retinoid X receptor. Cancer Res 65(19):8754–8765
73. Guillemin MC, Raffoux E, Vitoux D, Kogan S, Soilihi H et al (2002) In vivo activation of cAMP signaling induces growth arrest and differentiation in acute promyelocytic leukemia. J Exp Med 196(10):1373–1380
74. Lin RJ, Nagy L, Inoue S, Shao W, Miller WH Jr et al (1998) Role of the histone deacetylase complex in acute promyelocytic leukaemia. Nature 391(6669):811–814
75. Di Croce L, Raker VA, Corsaro M, Fazi F, Fanelli M et al (2002) Methyltransferase recruitment and DNA hypermethylation of target promoters by an oncogenic transcription factor. Science 295(5557):1079–1082
76. Villa R, Pasini D, Gutierrez A, Morey L, Occhionorelli M et al (2007) Role of the polycomb repressive complex 2 in acute promyelocytic leukemia. Cancer Cell 11(6):513–525
77. Vitaliano-Prunier A, Halftermeyer J, Ablain J, de Reynies A, Peres L, Le Bras M, Metzger D, de Thé H (2014) Clearance of PML/RARA-bound promoters suffice to initiate APL differentiation. Blood 124(25):3772–3780. doi:10.1182/blood-2014-03-561852
78. Santoro F, Botrugno OA, Dal Zuffo R, Pallavicini I, Matthews GM et al (2013) A dual role for Hdac1: oncosuppressor in tumorigenesis, oncogene in tumor maintenance. Blood 121(17):3459–3468
79. Leiva M, Moretti S, Soilihi H, Pallavicini I, Peres L et al (2012) Valproic acid induces differentiation and transient tumor regression, but spares leukemia-initiating activity in mouse models of APL. Leukemia 26(7):1630–1637
80. He LZ, Tolentino T, Grayson P, Zhong S, Warrell RP Jr et al (2001) Histone deacetylase inhibitors induce remission in transgenic models of therapy-resistant acute promyelocytic leukemia. J Clin Invest 108(9):1321–1330
81. Tabe Y, Konopleva M, Kondo Y, Contractor R, Jin L et al (2006) PML-RARalpha and AML1-ETO translocations are rarely associated with methylation of the RARbeta2 promoter. Ann Hematol 85(10):689–704
82. Wang K, Wang P, Shi J, Zhu X, He M et al (2010) PML/RARAlpha targets promoter regions containing PU.1 consensus and RARE half sites in acute promyelocytic leukemia. Cancer Cell 17(2):186–197
83. Zhu J, Zhou J, Peres L, Riaucoux F, Honore N et al (2005) A sumoylation site in PML/RARA is essential for leukemic transformation. Cancer Cell 7(2):143–153
84. Lallemand-Breitenbach V, de The H (2010) PML nuclear bodies. Cold Spring Harb Perspect Biol 2(5):a000661
85. Daniel MT, Koken M, Romagne O, Barbey S, Bazarbachi A et al (1993) PML protein expression in hematopoietic and acute promyelocytic leukemia cells. Blood 82(6):1858–1867
86. Koken MH, Puvion-Dutilleul F, Guillemin MC, Viron A, Linares-Cruz G et al (1994) The t(15;17) translocation alters a nuclear body in a retinoic acid-reversible fashion. EMBO J 13(5):1073–1083
87. Dyck JA, Maul GG, Miller WH Jr, Chen JD, Kakizuka A et al (1994) A novel macromolecular structure is a target of the promyelocyte-retinoic acid receptor oncoprotein. Cell 76(2):333–343
88. Zhu J, Koken MH, Quignon F, Chelbi-Alix MK, Degos L et al (1997) Arsenic-induced PML targeting onto nuclear bodies: implications for the treatment of acute promyelocytic leukemia. Proc Natl Acad Sci U S A 94(8):3978–3983

89. Sahin U, Ferhi O, Jeanne M, Benhenda S, Berthier C et al (2014) Oxidative stress-induced assembly of PML nuclear bodies controls sumoylation of partner proteins. J Cell Biol 204(6):931–945
90. Gambacorta M, Flenghi L, Fagioli M, Pileri S, Leoncini L et al (1996) Heterogeneous nuclear expression of the promyelocytic leukemia (PML) protein in normal and neoplastic human tissues. Am J Pathol 149(6):2023–2035
91. Terris B, Baldin V, Dubois S, Degott C, Flejou JF et al (1995) PML nuclear bodies are general targets for inflammation and cell proliferation. Cancer Res 55(7):1590–1597
92. Koken MH, Linares-Cruz G, Quignon F, Viron A, Chelbi-Alix MK et al (1995) The PML growth-suppressor has an altered expression in human oncogenesis. Oncogene 10(7):1315–1324
93. Matunis MJ, Zhang XD, Ellis NA (2006) SUMO: the glue that binds. Dev Cell 11(5):596–597
94. Pearson M, Carbone R, Sebastiani C, Cioce M, Fagioli M et al (2000) PML regulates p53 acetylation and premature senescence induced by oncogenic Ras. Nature 406(6792):207–210
95. Louria-Hayon I, Grossman T, Sionov RV, Alsheich O, Pandolfi PP et al (2003) The promyelocytic leukemia protein protects p53 from Mdm2-mediated inhibition and degradation. J Biol Chem 278(35):33134–33141
96. Bernardi R, Scaglioni PP, Bergmann S, Horn HF, Vousden KH et al (2004) PML regulates p53 stability by sequestering Mdm2 to the nucleolus. Nat Cell Biol 6(7):665–672
97. Vernier M, Bourdeau V, Gaumont-Leclerc MF, Moiseeva O, Begin V et al (2011) Regulation of E2Fs and senescence by PML nuclear bodies. Genes Dev 25(1):41–50
98. Occhionorelli M, Santoro F, Pallavicini I, Gruszka A, Moretti S et al (2011) The self-association coiled-coil domain of PML is sufficient for the oncogenic conversion of the retinoic acid receptor (RAR) alpha. Leukemia 25(5):814–820
99. Breitman TR, Collins SJ, Keene BR (1981) Terminal differentiation of human promyelocytic leukemic cells in primary culture in response to retinoic acid. Blood 57(6):1000–1004
100. Warrell RP Jr, Frankel SR, Miller WH Jr, Scheinberg DA, Itri LM et al (1991) Differentiation therapy of acute promyelocytic leukemia with tretinoin (all-trans-retinoic acid). N Engl J Med 324(20):1385–1393
101. Ablain J, Leiva M, Peres L, Fonsart J, Anthony E et al (2013) Uncoupling RARA transcriptional activation and degradation clarifies the bases for APL response to therapies. J Exp Med 210(4):647–653
102. Gurrieri C, Nafa K, Merghoub T, Bernardi R, Capodieci P et al (2004) Mutations of the PML tumor suppressor gene in acute promyelocytic leukemia. Blood 103(6):2358–2362
103. Tsimberidou AM, Tirado-Gomez M, Andreeff M, O'Brien S, Kantarjian H et al (2006) Single-agent liposomal all-trans retinoic acid can cure some patients with untreated acute promyelocytic leukemia: an update of The University of Texas M. D. Anderson Cancer Center Series. Leuk Lymphoma 47(6):1062–1068
104. Koken MH, Daniel MT, Gianni M, Zelent A, Licht J et al (1999) Retinoic acid, but not arsenic trioxide, degrades the PLZF/RARalpha fusion protein, without inducing terminal differentiation or apoptosis, in a RA-therapy resistant t(11;17)(q23;q21) APL patient. Oncogene 18(4):1113–1118
105. Guidez F, Parks S, Wong H, Jovanovic JV, Mays A et al (2007) RARalpha-PLZF overcomes PLZF-mediated repression of CRABPI, contributing to retinoid resistance in t(11;17) acute promyelocytic leukemia. Proc Natl Acad Sci U S A 104(47):18694–18699
106. Boylan JF, Gudas LJ (1991) Overexpression of the cellular retinoic acid binding protein-I (CRABP-I) results in a reduction in differentiation-specific gene expression in F9 teratocarcinoma cells. J Cell Biol 112(5):965–979
107. Rice KL, Hormaeche I, Doulatov S, Flatow JM, Grimwade D et al (2009) Comprehensive genomic screens identify a role for PLZF-RARalpha as a positive regulator of cell proliferation via direct regulation of c-MYC. Blood 114(27):5499–5511

108. Boukarabila H, Saurin AJ, Batsche E, Mossadegh N, van Lohuizen M et al (2009) The PRC1 polycomb group complex interacts with PLZF/RARA to mediate leukemic transformation. Genes Dev 23(10):1195–1206
109. Mathews V, George B, Chendamarai E, Lakshmi KM, Desire S et al (2010) Single-agent arsenic trioxide in the treatment of newly diagnosed acute promyelocytic leukemia: long-term follow-up data. J Clin Oncol 28(24):3866–3871
110. Chen GQ, Shi XG, Tang W, Xiong SM, Zhu J et al (1997) Use of arsenic trioxide (As2O3) in the treatment of acute promyelocytic leukemia (APL): I. As2O3 exerts dose-dependent dual effects on APL cells. Blood 89(9):3345–3353
111. Zhu Q, Zhang JW, Zhu HQ, Shen YL, Flexor M et al (2002) Synergic effects of arsenic trioxide and cAMP during acute promyelocytic leukemia cell maturation subtends a novel signaling cross-talk. Blood 99(3):1014–1022
112. Muto A, Kizaki M, Kawamura C, Matsushita H, Fukuchi Y et al (2001) A novel differentiation-inducing therapy for acute promyelocytic leukemia with a combination of arsenic trioxide and GM-CSF. Leukemia 15(8):1176–1184
113. Jeanne M, Lallemand-Breitenbach V, Ferhi O, Koken M, Le Bras M et al (2010) PML/RARA oxidation and arsenic binding initiate the antileukemia response of As2O3. Cancer Cell 18(1):88–98
114. Zhang XW, Yan XJ, Zhou ZR, Yang FF, Wu ZY et al (2010) Arsenic trioxide controls the fate of the PML-RARalpha oncoprotein by directly binding PML. Science 328(5975):240–243
115. Lallemand-Breitenbach V, Zhu J, Puvion F, Koken M, Honore N et al (2001) Role of promyelocytic leukemia (PML) sumolation in nuclear body formation, 11S proteasome recruitment, and As2O3-induced PML or PML/retinoic acid receptor alpha degradation. J Exp Med 193(12):1361–1371
116. Lallemand-Breitenbach V, Jeanne M, Benhenda S, Nasr R, Lei M et al (2008) Arsenic degrades PML or PML-RARalpha through a SUMO-triggered RNF4/ubiquitin-mediated pathway. Nat Cell Biol 10(5):547–555
117. Tatham MH, Geoffroy MC, Shen L, Plechanovova A, Hattersley N et al (2008) RNF4 is a poly-SUMO-specific E3 ubiquitin ligase required for arsenic-induced PML degradation. Nat Cell Biol 10(5):538–546
118. Goto E, Tomita A, Hayakawa F, Atsumi A, Kiyoi H et al (2011) Missense mutations in PML-RARA are critical for the lack of responsiveness to arsenic trioxide treatment. Blood 118(6):1600–1609
119. Zhu HH, Qin YZ, Huang XJ (2014) Resistance to arsenic therapy in acute promyelocytic leukemia. N Engl J Med 370(19):1864–1866
120. Zhu J, Gianni M, Kopf E, Honore N, Chelbi-Alix M et al (1999) Retinoic acid induces proteasome-dependent degradation of retinoic acid receptor alpha (RARalpha) and oncogenic RARalpha fusion proteins. Proc Natl Acad Sci U S A 96(26):14807–14812
121. Nervi C, Ferrara FF, Fanelli M, Rippo MR, Tomassini B et al (1998) Caspases mediate retinoic acid-induced degradation of the acute promyelocytic leukemia PML/RARAlpha fusion protein. Blood 92(7):2244–2251
122. Isakson P, Bjoras M, Boe SO, Simonsen A (2010) Autophagy contributes to therapy-induced degradation of the PML/RARA oncoprotein. Blood 116(13):2324–2331
123. Zhu J, Lallemand-Breitenbach V, de The H (2001) Pathways of retinoic acid- or arsenic trioxide-induced PML/RARAlpha catabolism, role of oncogene degradation in disease remission. Oncogene 20(49):7257–7265
124. Zheng PZ, Wang KK, Zhang QY, Huang QH, Du YZ et al (2005) Systems analysis of transcriptome and proteome in retinoic acid/arsenic trioxide-induced cell differentiation/apoptosis of promyelocytic leukemia. Proc Natl Acad Sci U S A 102(21):7653–7658
125. Lallemand-Breitenbach V, Guillemin MC, Janin A, Daniel MT, Degos L et al (1999) Retinoic acid and arsenic synergize to eradicate leukemic cells in a mouse model of acute promyelocytic leukemia. J Exp Med 189(7):1043–1052

126. Rego EM, He LZ, Warrell RP Jr, Wang ZG, Pandolfi PP (2000) Retinoic acid (RA) and As2O3 treatment in transgenic models of acute promyelocytic leukemia (APL) unravel the distinct nature of the leukemogenic process induced by the PML-RARalpha and PLZF-RARalpha oncoproteins. Proc Natl Acad Sci U S A 97(18):10173–10178
127. Shen ZX, Shi ZZ, Fang J, Gu BW, Li JM et al (2004) All-trans retinoic acid/As2O3 combination yields a high quality remission and survival in newly diagnosed acute promyelocytic leukemia. Proc Natl Acad Sci U S A 101(15):5328–5335
128. Hasegawa H, Sawa H, Lewis MJ, Orba Y, Sheehy N et al (2006) Thymus-derived leukemia-lymphoma in mice transgenic for the Tax gene of human T-lymphotropic virus type I. Nat Med 12(4):466–472
129. El-Sabban ME, Nasr R, Dbaibo G, Hermine O, Abboushi N et al (2000) Arsenic-interferon-alpha-triggered apoptosis in HTLV-I transformed cells is associated with tax down-regulation and reversal of NF-kappa B activation. Blood 96(8):2849–2855
130. El Hajj H, El-Sabban M, Hasegawa H, Zaatari G, Ablain J et al (2010) Therapy-induced selective loss of leukemia-initiating activity in murine adult T cell leukemia. J Exp Med 207(13):2785–2792
131. Kchour G, Tarhini M, Kooshyar MM, El Hajj H, Wattel E et al (2009) Phase 2 study of the efficacy and safety of the combination of arsenic trioxide, interferon alpha, and zidovudine in newly diagnosed chronic adult T-cell leukemia/lymphoma (ATL). Blood 113(26):6528–6532

Chapter 13
Chromosome Abnormalities in Acute Myeloid Leukaemia and Their Clinical Importance

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Contents

13.1 Introduction 276
13.2 General Cytogenetic Characteristics of AML 276
13.3 Chromosome Abnormalities Denoting Separate Entities in the WHO Classification of AML 284
13.3.1 AML with t(8;21)(q22;q22)/*RUNX1-RUNX1T1* 284
13.3.2 AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22)/*CBFB-MYH11* 287
13.3.3 AML with t(15;17)(q22;q12); *PML-RARA* 288
13.3.4 AML with t(9;11)(p22;q23); *KMT2A(MLL)-MLLT3* 290
13.3.5 AML with t(6;9)(p23;q34); *DEK-NUP214* 291
13.3.6 AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1(MECOM)* 291
13.3.7 AML with t(1;22)(p13;q13); *RBM15-MKL1* 292
13.4 AML with Myelodysplasia-Related Changes 292
13.5 Correlations Between Cytogenetic Findings and Clinical Outcome of AML Patients 293
13.6 Concluding Remarks and Future Directions 305
Acknowledgements 306
References 306

Abstract Acute myeloid leukaemia (AML) is a neoplastic disease developing as a result of accumulation of somatic genetic alterations, both detectable microscopically as structural and numerical chromosome abnormalities and submicroscopic, such as gene mutations and changes in gene and microRNA expression, in haematopoietic progenitor cells. The cytogenetic landscape of AML is very heterogeneous with more than 300 recurrent abnormalities identified to date. Several of these abnormalities are now used to delineate separate disease entities in the World Health Organization Classification of AML. Moreover, pretreatment cytogenetic findings are among the most important, independent prognostic factors in both adults and children with AML.

Keywords Acute myeloid leukaemia • Cytogenetics • Mutation • Leukaemia diagnosis • Prognostication

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13.1 Introduction

Cytogenetic analyses of leukaemic blasts from patients diagnosed with acute myeloid leukaemia (AML) have been instrumental in determining the genetic basis of this disease, and greatly increased our understanding of its extraordinary histopathologic, immunophenotypic and clinical heterogeneity. Since 1973, when Janet D. Rowley employed the then newly introduced chromosome Q-banding technique [1] to describe the t(8;21) (q22;q22) [2], the translocation that turned out to be one of the more frequent and clinically important chromosome abnormalities in AML, more than 300 numerical and structural abnormalities, both balanced and unbalanced, have been identified as recurring [3–5]. Importantly, molecular dissection of breakpoints in numerous AML-associated translocations and inversions has led to cloning of genes that play a pivotal role in leukaemogenesis through deregulation of differentiation, proliferation and/or apoptosis (programmed cell death) of haematopoietic progenitor cells [6, 7]. However, it has become clear that a single cytogenetic abnormality is usually not sufficient to cause overt AML, whose development is usually a result of acquisition of multiple somatic alterations affecting different pathways within the same cell [8]. These include mutations activating genes involved in signal transduction of proliferation pathways that bestow a survival advantage and cause increased rate of cell proliferation. The other kind of leukaemia-associated rearrangements, often occurring in the form of intragenic mutations or gene fusions generated by reciprocal chromosome translocations or inversions, disrupt genes involved in the process of normal cell differentiation [9].

Even though abnormalities detectable using cytogenetic methodology constitute only a fraction of acquired genetic alterations in AML, chromosome abnormalities, both those that have been characterized molecularly and those that have not, constitute tumour markers with diagnostic and prognostic significance. Thus, several chromosome abnormalities and their molecular counterparts are now used to delineate separate disease entities in the World Health Organization (WHO) Classification of Tumours of Haematopoietic and Lymphoid Tissues [10]. Moreover, pretreatment karyotype has been repeatedly shown to be among the most important, independent prognostic factors for achievement of complete remission (CR), and duration of disease-free (DFS) and overall (OS) survival in both adults [11–23] and children with AML [24–26].

In this chapter, after a brief overview of general cytogenetic features of AML, we will present the role that cytogenetic findings play in establishing diagnosis of distinct disease subsets, and then discuss correlations between karyotype and clinical outcome of patients with AML.

13.2 General Cytogenetic Characteristics of AML

Following short-term, i.e., 24- or 48-h, unstimulated in vitro culture of bone marrow (or, less preferably, blood) samples aspirated before the start of therapy, sufficient numbers of good quality metaphase cells are obtained in a vast majority of patients,

with failure rates usually below 10 % [3, 12, 14, 23, 27]. Unsuccessful pretreatment cytogenetic investigation has been reported to portend an adverse prognosis in a recent study, in which 6 % of almost 1,500 AML patients whose samples were subjected to standard chromosome analysis had results deemed to be unacceptable [28]. Similarly, cytogenetic analysis failed in 7 % of over 700 children treated on the United Kingdom Medical Research Council (MRC) protocols [25].

Among patients with de novo AML, an abnormal karyotype containing one or more clonal chromosomal aberrations, namely, the same structural alteration or an extra copy of the same chromosome (trisomy) present in ≥2 metaphases or a loss of the same chromosome (monosomy) seen in ≥3 metaphases, is detected in 55–61 % of adult [14–16, 22, 23] and 76–78 % of paediatric cases [25, 26, 29, 30]. Although the reasons for this age discrepancy are unknown, they likely reflect biologic differences between adult and paediatric disease, as exemplified by age-related disparities in the incidence of specific chromosome abnormalities. For instance, reciprocal translocations, insertions and inversions involving chromosome band 11q23 that lead to rearrangements of the *KMT2A* gene (formerly known as *MLL* [31]) are approximately four times more frequent in children than in adults [17]. Moreover, their frequency diminishes substantially with age, being the highest, 51–58 %, in infants with AML below the age of 12 months [32–34], followed by 39 % of children aged from 13 to 24 months [35], 8–9 % of children older than 24 months [30, 35], and 4–7 % of adults [15, 16, 22], among whom only ≤3 % of those aged 60 years or older harbour 11q23/*KMT2A(MLL)* alterations [19, 20]. Likewise, a cryptic, i.e., not detectable by a routine cytogenetic study, translocation (5;11)(q35.2;p15.4), creating the *NUP98-NSD1* fusion gene, is found seven times more often in paediatric as opposed to adult AML patients [36]. Additionally, there are rare abnormalities that have been hitherto detected in children only. These include another cryptic translocation, t(7;12)(q36;p13), resulting in the *MNX1-ETV6* fusion gene [37, 38], and the t(1;22)(p13;q13), creating the *RBM15-MKL1* fusion gene [39]. The latter translocation has been hitherto found almost exclusively in young children under the age of 24 months [5, 39].

Conversely, t(8;21) and t(15;17)(q22-24;q12-21), the two most frequent translocations in both older children and adults diagnosed with AML [17], have not been detected in infants younger than 12 months [26, 35], although the incidence of t(8;21) is twice as high in older children as it is in adults. In contrast, inv(3)(q21;q26.2) and t(3;3)(q21;q26.2), as well as del(5q) and other unbalanced structural abnormalities leading to loss of material from the long arm of chromosome 5 (5q), are more frequent in adult rather than paediatric AML, as is complex karyotype with five or more chromosome aberrations. Finally, the incidence of certain abnormalities, such as inv(16)(p13.1q22) or t(16;16)(p13.1;q22), or trisomy of chromosome 8 (+8) is comparable in childhood and adult AML [5, 17].

Based on presumed significance, recurrent chromosome aberrations have been separated into primary and secondary ones. The former are considered to be most important and deemed to play an essential role in the early stages of leukaemogenesis. Primary abnormalities are quite specific for AML; that is, they are seldom (or never) found in other types of haematologic neoplasms or solid tumours, and can be found as the only microscopically detectable rearrangements in some patients [40].

They may have a profound influence on clinical characteristics of patients that harbour them, and thus several primary abnormalities have been chosen to denote distinct disease categories of AML (please see Sect. 13.3 below). Balanced rearrangements, such as reciprocal translocations, insertions and inversions, with presumed primary significance, which are currently known to be recurrent, are presented in Table 13.1 together with the genes they alter (whenever known) and the numbers of patients reported to carry these abnormalities in the literature and indexed in the Mitelman Database [5].

In addition to balanced abnormalities, a number of unbalanced aberrations, e.g., deletions, unbalanced translocations and isochromosomes, have been detected recurrently as a sole abnormality in AML patients, and consequently might represent rearrangements of primary importance in these patients. The most frequent of those are del(5q), del(7q), del(9q), del(12p), del(20q) and del(13q) [3, 5]. In contrast to reciprocal translocations or inversions, which result in rearrangements of specific genes, the molecular consequences of recurrent deletions do not seem to be restricted to a loss of and/or mutation in a single tumour suppressor gene, but rather to haploinsufficiency of multiple genes located in a deleted chromosome segment, i.e., diminished gene expression caused by the presence of only one functional allele remaining after a deletion of the second allele. However, it is possible that some of the deleted and/or underexpressed genes may still be more important than others, and recent studies have identified *CTNNA1* [41] and *EGR1* [42] as candidate tumour suppressor genes (TSGs) in AML with del(5q), and *CUX1* [43] and *KMT2C* (*MLL3*) [44] as TSGs in AML with del(7q).

Numerical chromosome changes can also be considered of potential primary import when they are found as the only cytogenetic alteration. The most frequent isolated numerical aberration in AML is +8, detected in ~4 % of adults with de novo AML, followed by recurrent, albeit less frequent, monosomy 7 (−7; 1 %), +11 (1 %), +13 (1 %), loss of chromosome Y (–Y; 1 %), +21 (0.5 %), and +4 (0.3 %) [45]. The molecular mechanisms whereby recurrent trisomies contribute to leukaemogenesis are mostly unknown. To date, only isolated +11 and +13 have been linked with particular molecular defects occurring in a high proportion of patients with these trisomies, namely, a partial tandem duplication of the *KMT2A(MLL)* gene [*KMT2A(MLL)*-PTD] associated with +11 [46] and *RUNX1* mutations associated with +13 [47]. Recently patients with +8 were shown to harbour recurrent gene mutations but the most frequent of them were each detected in ~30 % of patients [mutations in *RUNX1* and *ASXL1*, and the internal tandem duplication of the *FLT3* gene (*FLT3*-ITD)]; also common were *IDH2*, *DNMT3A* and *NPM1* mutations found in around 25 % of the patients [48].

Secondary abnormalities, which can accompany a primary abnormality either in all cells or be present in only a fraction of cells that harbour a primary aberration, are predominantly unbalanced, and include both numerical (trisomy, monosomy) and structural alterations (deletion, unbalanced translocation) [49]. Secondary abnormalities are generally less specific, and the same one can be recurrently found together with diverse primary aberrations in AML as well as in other types of leukaemia or even in non-haematologic malignancies. A prime example of such an

Table 13.1 Chromosome aberrations of presumed primary significance in AML

Cytogenetic abnormality[a]	Gene(s) involved	Number of AML cases with the abnormality[b]	No. (%) of AML cases with the abnormality occurring as a sole alteration[b]
Rearrangements involving band 1p36 and the *PRDM16* gene			
t(1;3)(p36.3;q21.3)	*RPN1-PRDM16*	49	32 (**65**%)
t(1;17)(p36;q21)	*PRDM16*	3	2 (**67**%)
Rearrangement involving band 1p36			
t(1;7)(p36;q34)	–	3	2 (**67**%)
Rearrangement involving band 1p13 and the *RBM15* gene			
t(1;22)(p13;q13)	*RBM15-MKL1*	41	33 (**80**%)
Rearrangement involving band 2p23			
t(2;4)(p23;q25)	–	2	2 (**100**%)
Rearrangement involving band 2p13			
t(2;12)(p13;p13)	–	3	1 (**33**%)
Rearrangement involving band 3p21			
t(3;7)(p21;q35)	–	4	2 (**50**%)
Rearrangements involving band 3q26 and the *MECOM* (*EVI1*) gene			
t(2;3)(p15-21;q26-27)	*MECOM*	22	10 (**45**%)
inv(3)(q21q26.2)	*RPN1-MECOM*	306	111 (**36**%)
t(3;3)(q21;q26.2)[c]	*RPN1-MECOM*	139	62 (**45**%)
t(3;8)(q26;q24)	*MECOM*	9	4 (**44**%)
Rearrangements involving band 5q31			
t(5;6)(q31;q21)	–	3	3 (**100**%)
t(5;21)(q31;q22)	–	2	1 (**50**%)
Rearrangement involving band 5q35 and the *NPM1* gene			
t(3;5)(q25;q35)[d]	*MLF1-NPM1*	71	58 (**82**%)
Rearrangement involving band 6q23 and the *MYB* gene			
t(X;6)(p11;q23)	*MYB-GATA1*	4	4 (**100**%)
Rearrangements involving band 8p11 and the *KAT6A* gene			
inv(8)(p11q13)	*KAT6A-NCOA2*	7	6 (**86**%)
t(8;16)(p11;p13)	*KAT6A-CREBBP*	115	68 (**59**%)
t(8;19)(p11;q13.3)	*KAT6A*	3	3 (**100**%)
t(8;22)(p11;q13)	*KAT6A-EP300*	4	3 (**75**%)
Rearrangements involving band 8p11 and the *FGFR1*gene			
t(6;8)(q27;p11)	*FGFR1OP-FGFR1*	4	3 (**75**%)
t(8;9)(p11;q33)	*CNTRL-FGFR1*	2	0
Rearrangement involving band 8q24			
t(8;14)(q24;q32)	–	5	1 (**20**%)
Rearrangement involving band 9p24 and the *JAK2* gene			
t(8;9)(p22;p24)	*PCM1-JAK2*	4	2 (**50**%)
Rearrangement involving band 9q34 and the *NUP214* gene			
t(6;9)(p23;q34)	*DEK-NUP214*	94	78 (**83**%)

(continued)

Table 13.1 (continued)

Cytogenetic abnormality[a]	Gene(s) involved	Number of AML cases with the abnormality[b]	No. (%) of AML cases with the abnormality occurring as a sole alteration[b]
Rearrangement involving band 9q34 and the *ABL1* gene			
t(9;22)(q34;q11.2)	*BCR-ABL1*	230	94 (**41**%)
Rearrangement involving band 9q34			
t(9;13)(q34;q12)	–	2	2 (**100**%)
Rearrangement involving band 10q22 and the *KAT6B* gene			
t(10;16)(q22;p13)	*KAT6B-CREBBP*	2	1 (**50**%)
Rearrangements involving band 11p15 and the *NUP98* gene			
t(1;11)(q24;p15)	*NUP98-PRRX1*	3	1 (**33**%)
t(2;11)(q31;p15)	*NUP98-HOXD11* or *NUP98-HOXD13*	6	5 (**83**%)
t(3;11)(p11;p15)	*NUP98-POU1F1*	2	2 (**100**%)
t(3;11)(q12;p15)	*NUP98-LNP1*	3	3 (**100**%)
t(4;11)(q23;p15)	*NUP98-RAP1GDS1*	4	2 (**50**%)
t(5;11)(q35.2;p15.4)	*NUP98-NSD1* or *STIM1-NSD1*	34	26 (**77**%)
t(7;11)(p15;p15)	*HOXA9-NUP98* or *HOXA11-NUP98* or *HOXA13-NUP98*	67	58 (**87**%)
t(7;11)(q22;p15)	*NUP98*	3	2 (**67**%)
t(8;11)(p11;p15)	*NUP98-WHSC1L1*	5	2 (**40**%)
t(9;11)(p22;p15)	*NUP98-PSIP1*	5	5 (**100**%)
t(9;11)(q34;p15)	*NUP98-PRRX2*	2	2 (**50**%)
t(10;11)(q23;p15)	*NUP98-HHEX*	4	2 (**50**%)
inv(11)(p15q22)	*NUP98-DDX10*	10	7 (**70**%)
t(11;11)(p15;q22)	*NUP98-DDX10*	2	2 (**100**%)
inv(11)(p15q23)	*NUP98-KMT2A(MLL)*	13	9 (**69**%)
t(11;12)(p15;p13)[e]	*NUP98-KDM5A*	6	1 (**17**%)
t(11;12)(p15;q13)	*NUP98-HOXC11* or *NUP98-HOXC13*	13	12 (**92**%)
t(11;17)(p15;q21)	*NUP98*	3	3 (**100**%)
t(11;17)(p15;q23)	*NUP98*	3	0
t(11;20)(p15;q12)	*NUP98-TOP1*	13	10 (**77**%)
Rearrangement involving band 11p15			
t(X;11)(q13;p15)	–	3	1 (**33**%)
Rearrangements involving band 11q13			
t(9;11)(p22;q13)	–	6	6 (**100**%)
t(11;12)(q13;p13)	–	4	1 (**25**%)
Rearrangement involving band 11q14 and the *PICALM* gene			
t(10;11)(p12;q14)	*MLLT10-PICALM*	72	36 (**50**%)
Rearrangements involving band 11q23 and the *KMT2A(MLL)* gene			
t(X;11)(q13;q23)	*KMT2A(MLL)-FOXO4*	3	3 (**100**%)

(continued)

Table 13.1 (continued)

Cytogenetic abnormality[a]	Gene(s) involved	Number of AML cases with the abnormality[b]	No. (%) of AML cases with the abnormality occurring as a sole alteration[b]
t(X;11)(q24;q23)	*KMT2A(MLL)-SEPT6*	4	4 (**100**%)
ins(11;X)(q23;q28q12)	*KMT2A(MLL)-FLNA*	2	2 (**100**%)
t(1;11)(p32;q23)	*KMT2A(MLL)-EPS15*	11	6 (**55**%)
t(1;11)(q21;q23)	*KMT2A(MLL)-MLLT11*	26	20 (**77**%)
t(2;11)(p21;q23)	*KMT2A(MLL)*	22	11 (**50**%)
t(2;11)(q37;q23)	*KMT2A(MLL)-SEPT2*	6	3 (**50**%)
t(4;11)(q21;q23)	*KMT2A(MLL)-AFF1*	29	18 (**62**%)
t(5;11)(q31;q23)	*KMT2A(MLL)-ARHGAP26*	5	5 (**100**%)
t(6;11)(q15;q23)	*KMT2A(MLL)-CASP8AP2*	3	2 (**67**%)
t(6;11)(q21;q23)	*KMT2A(MLL)-FOXO3*	5	2 (**40**%)
t(6;11)(q27;q23)	*KMT2A(MLL)-MLLT4*	93	83 (**89**%)
t(9;11)(p22;q23)	*KMT2A(MLL)-MLLT3*	264	177 (**67**%)
t(9;11)(q21;q23)	*KMT2A(MLL)*	3	3 (**100**%)
t(9;11)(q22;q23)	*KMT2A(MLL)*	6	5 (**83**%)
ins(10;11)(p11-13;q23q13-25)	*KMT2A(MLL)-MLLT10*	35	19 (**54**%)
ins(11;10)(q23;p12-13p11-15)	*KMT2A(MLL)-MLLT10*	4	2 (**50**%)
t(10;11)(p12;q13-23)	*KMT2A(MLL)-MLLT10*	47	28 (**60**%)
t(10;11)(p12;q23)	*KMT2A(MLL)-ABI1*	3	3 (**100**%)
t(10;11)(q21;q23)	*KMT2A(MLL)-TET1*	5	2 (**40**%)
t(11;11)(q13;q23)	*KMT2A(MLL)-ARHGEF17*	3	2 (**67**%)
del(11)(q23q23)[f]	*KMT2A(MLL)-ARHGEF12*	2	0
t(11;12)(q23;q13)	*KMT2A(MLL)-SARPN*	2	2 (**100**%)
t(11;14)(q23;q23)	*KMT2A(MLL)-GPHN*	3	3 (**100**%)
t(11;14)(q23;q32)	*KMT2A(MLL)-CEP170B*	3	3 (**100**%)
t(11;15)(q23;q15)	*KMT2A(MLL)-CASC5* or *KMT2A(MLL)-ZFYVE19*	8	1 (**13**%)
t(11;16)(q23;p13.3)	*KMT2A(MLL)-CREBBP*	10	5 (**50**%)
t(11;17)(q23;p13.1)	*KMT2A(MLL)-GAS7*	2	?1 (?**50**%)[g]
t(11;17)(q23;q12-21)	*KMT2A(MLL)-MLLT6*	42	34 (**81**%)
t(11;17)(q23;q23)	*KMT2A(MLL)*	4	2 (**50**%)
t(11;17)(q23;q25)	*KMT2A(MLL)-SEPT9*	34	22 (**65**%)[h]
t(11;19)(q23;p13.1)	*KMT2A(MLL)-ELL*	69	58 (**85**%)[h]
t(11;19)(q23;p13.3)	*KMT2A(MLL)-MLLT1*	47	19 (**41**%)
t(11;19)(q23;p13.2-13.3)	*KMT2A(MLL)-MYO1F*	4	1 (**25**%)
t(11;22)(q23;q11)	*KMT2A(MLL)-SEPT5*	8	5 (**63**%)
t(11;22)(q23;q13)	*KMT2A(MLL)-EP300*	6	4 (**67**%)

(continued)

Table 13.1 (continued)

Cytogenetic abnormality[a]	Gene(s) involved	Number of AML cases with the abnormality[b]	No. (%) of AML cases with the abnormality occurring as a sole alteration[b]
Rearrangements involving band 12p13 and the *ETV6* gene			
t(1;12)(q25;p13)	*ETV6-ABL2*	2	1 (**50**%)[i]
t(3;12)(q26;p13)	*ETV6-MECOM*	36	23 (**64**%)
t(4;12)(q12;p13)	*CHIC2-ETV6*	25	17 (**68**%)
t(5;12)(q13;p13)	*ETV6*	3	2 (**67**%)
t(5;12)(q31;p13)	*ACSL6-ETV6*	4	2 (**50**%)
t(7;12)(p15;p13)	*ETV6*	7	1 (**14**%)
t(7;12)(q36;p13)	*MNX1-ETV6*	20	2 (**10**%)
t(10;12)(q24;p13)	*ETV6-GOT1*	6	2 (**33**%)
inv(12)(p13q24)	*ETV6*	2	0
t(12;12)(p13;q13)	*ETV6*	3	0
t(12;13)(p13;q12)	*ETV6-CDX2* or *FLT3-ETV6*	4	2 (**50**%)
t(12;22)(p12-13;q11-13)	*ETV6-MN1*	22	5 (**23**%)
Rearrangements involving band 16q22 and the *CBFB* gene			
inv(16)(p13.1q22)	*MYH11-CBFB*	848	581 (**69**%)
t(16;16)(p13.1;q22)	*MYH11-CBFB*	45	37 (**82**%)
Rearrangements involving band 16q24 and the *CBFA2T3* gene			
inv(16)(p13.3q24.3)[j]	*CBFA2T3-GLIS2*	32	22 (**69**%)
t(16;21)(q24.3;q22)	*CBFA2T3-RUNX1*	23	6 (**26**%)
Rearrangement involving band 17q11 and the *TAF15* gene			
t(12;17)(p13;q11-12)	*TAF15-ZNF384*	4	4 (**100**%)
Rearrangements involving bands 17q12-21 and the *RARA* gene			
t(4;17)(q12;q21)	*FIP1L1-RARA*	2	0
t(5;17)(q35;q12-21)	*NPM1-RARA*	4	1 (**25**%)
t(11;15;17)(q13;q22;q12)	*PML-RARA*	2	2 (**100**%)
t(11;17)(q23;q21)	*ZBTB16-RARA*	11	7 (**64**%)
t(15;17)(q22-24;q12-21)[k]	*PML-RARA*	1171	843 (**72**%)
ins(15;17)(q22;q21q21)	*PML-RARA*	5	0
Rearrangements involving band 21q22 and the *RUNX1* gene			
t(1;21)(p36;q22)	*RUNX1-PRDM16*	2	2 (**100**%)
t(1;21)(p22;q22)	*RUNX1-CLCA2*	2	2 (**100**%)
t(3;21)(q26.2;q22.1)	*MECOM-RUNX1* or *RUNX1-RPL22P1*	63	30 (**48**%)
t(7;21)(p22;q22)	*RUNX1-USP42*	5	3 (**60**%)
t(8;21)(q22;q22)	*RUNX1-RUNX1T1*	1569	676 (**43**%)
ins(8;21)(q22;q22q22)	*RUNX1-RUNX1T1*	7	3 (**43**%)
ins(21;8)(q22;q13q22)	*RUNX1-RUNX1T1*	2	1 (**50**%)

(continued)

Table 13.1 (continued)

Cytogenetic abnormality[a]	Gene(s) involved	Number of AML cases with the abnormality[b]	No. (%) of AML cases with the abnormality occurring as a sole alteration[b]
ins(21;8)(q22;q21q22)	*RUNX1-RUNX1T1*	4	4 (**100**%)
ins(21;8)(q22;q22q22)	*RUNX1-RUNX1T1*	5	2 (**40**%)
t(1;8;21)(p13;q22;q22)	*RUNX1-RUNX1T1*	2	1 (**50**%)
t(5;8;21)(q31;q22;q22)	*RUNX1-RUNX1T1*	2	2 (**100**%)
t(8;10;21)(q22;q24;q22)	*RUNX1-RUNX1T1*	2	1 (**50**%)
t(8;12;21)(q22;p13;q22)	*RUNX1-RUNX1T1*	2	1 (**50**%)
t(8;12;21)(q22;q13;q22)	*RUNX1-RUNX1T1*	3	3 (**100**%)
t(8;15;21)(q22;q21;q22)	*RUNX1-RUNX1T1*	2	1 (**50**%)
t(8;17;21)(q22;q23;q22)	*RUNX1-RUNX1T1*	3	1 (**33**%)
t(8;20;21)(q22;q13;q22)	*RUNX1-RUNX1T1*	2	1 (**50**%)
t(11;21)(q13;q22)	*RUNX1-MACROD1*	2	1 (**50**%)
t(17;21)(q11.2;q22)	*RUNX1*	3	0
t(18;21)(q21;q22)	*RUNX1*	3	1 (**33**%)
Rearrangement involving band 21q22 and the *ERG* gene			
t(16;21)(p11;q22)	*FUS-ERG*	58	39 (**67**%)
Rearrangements involving band 21q22			
t(5;21)(q13;q22)	–	3	2 (**67**%)
t(6;21)(q13;q22)	–	4	1 (**25**%)
Rearrangement involving band Xp11			
t(X;10)(p11;p11)	–	4	1 (**25**%)

Data from Mitelman et al. [5]

[a]Chromosome aberrations disrupting the same chromosome band and the same gene, if known, are grouped together. Within a given group, aberrations are arranged according to the numerical order of the first chromosome involved. Each aberration is presented only once in the Table

[b]Number of cases with a given abnormality indexed in the Mitelman Database [5]

[c]Also interpreted as ins(3;3)(q21;q21q26)

[d]Also interpreted as t(3;5)(q21;q31)

[e]This translocation is cryptic; there are no individual cases listed in the Mitelman Database [5]. The numbers of cases listed in the Table are based on data from van Zutven et al. [183], Hollink et al. [36], and Gruber et al. [128]

[f]This deletion is cryptic; there are no individual cases listed in the Mitelman Database [5]. The numbers of cases listed in the Table are based on data from Kourlas et al. [184] and Shih et al. [185]

[g]For one patient with *KMT2A(MLL)-GAS7* fusion, the karyotype description was not available [88]

[h]In one case, it is unknown whether t(11;19) was a sole abnormality or not. This case was not included in calculation of a percentage of cases with sole t(11;19)

[i]inv(16) was present in one case and a cryptic rearrangement resulting in the *CBFB-MYH11* gene fusion was detected in the other

[j]This inversion is cryptic; there are no individual cases listed in the Mitelman Database [5]. The numbers of cases listed in the Table are based on data from Gruber et al. [128] and Masetti et al. [129]

[k]In the literature, the breakpoints in t(15;17) have been variously assigned to 15q22 or 15q24, and to 17q11, 17q12, 17q21 or 17q22. Based on the human genome sequence, the breakpoints are currently defined as 15q24.1 and 17q21.1

ubiquitous secondary aberration is trisomy of chromosome 8, which can be found not only in AML patients with such primary aberrations as t(6;9)(p23;q34), t(9;11)(p22;q23), t(9;22)(q34;q11.2), t(15;17) or inv(16)/t(16;16) but also in patients diagnosed with myelodysplastic syndromes (MDS), acute lymphoblastic leukaemia (ALL), non-Hodgkin lymphoma and several types of solid tumours, including Ewing sarcoma, myxoid liposarcoma, synovial sarcoma or clear cell sarcoma of tendons and aponeuroses [5]. Similarly widespread among various types of neoplasia, albeit less frequent, is an unbalanced structural abnormality, der(16)t(1;16)(q12-23;q12-24) [50]. Conversely, other secondary aberrations, e.g., loss of the entire chromosome X (–X), del(5q) or del(9q), seem to be much more specific for AML [49]. Notably, some primary abnormalities are predisposed to occur together with secondary changes more often than others. For instance, one or more secondary aberrations are detected in ~70 % of patients with either t(8;21) or inv(3)/t(3;3), whereas this is the case in only one-third of patients with t(15;17) or those with inv(16) and ~10 % of t(6;9)-positive patients [5, 51–55].

13.3 Chromosome Abnormalities Denoting Separate Entities in the WHO Classification of AML

Specific chromosome abnormalities together with their molecular counterparts were first used to identify separate disease entities in the 2001 revision of the WHO classification [56]. The latest 2008 revision increased the role of the cytogenetic findings and molecular genetics for disease categorization, and recognized seven separate groups within the "AML with recurrent genetic abnormalities" category (Table 13.2 and Fig. 13.1) [10]. Below, we will briefly review each of these groups.

13.3.1 AML with t(8;21)(q22;q22)/RUNX1-RUNX1T1

This is one of the two AML entities that together constitute core binding factor (CBF) AML. CBF-AML is so named because the genes rearranged by chromosome aberrations, i.e., *RUNX1*, located at 21q22 and disrupted by t(8;21)(q22;q22), and *CBFB*, located at 16q22 and disrupted by inv(16)(p13.1q22) or t(16;16)(p13.1;q22), encode, respectively, the alpha and beta subunits of the CBF complex, which is a heterodimeric transcription factor regulating transcription of genes encoding proteins involved in haematopoietic differentiation, such as interleukin-3, neutrophil elastase, macrophage colony-stimulating factor receptor or granulocyte-macrophage colony-stimulating factor [57]. Juxtaposition of 8q22 and 21q22 loci by the t(8;21) or its variants creates a chimeric *RUNX1-RUNX1T1* gene whose protein product is capable of a dominant-negative inhibition of the wild-type RUNX1 and impairment of normal haematopoiesis.

Table 13.2 Specific chromosomal alterations used to define AML entities in the WHO classification of tumours of haematopoietic and lymphoid tissues

Chromosome abnormalities and the related gene rearrangements denoting specific entities within the WHO category of AML entitled "AML with recurrent genetic abnormalities"
t(8;21)(q22;q22); *RUNX1-RUNX1T1*
inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
t(15;17)(q22;q12); *PML-RARA*
t(9;11)(p22;q23); *MLLT3-MLL*
t(6;9)(p23;q34); *DEK-NUP214*
inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*
t(1;22)(p13;q13); *RBM15-MKL1*
Chromosome abnormalities whose detection allows diagnosis of "AML with myelodysplasia-related changes"
Complex karyotype[a]
Unbalanced abnormalities
−7 or del(7q)
−5 or del(5q)
i(17q) or t(17p)
−13 or del(13q)
del(11q)
del(12p) or t(12p)
del(9q)
idic(X)(q13)
Balanced abnormalities
t(11;16)(q23;p13.3)[b]
t(3;21)(q26.2;q22.1)[b]
t(1;3)(p36.3;q21.1)
t(2;11)(p21;q23)[b]
t(5;12)(q32;p12)
t(5;7)(q32;q11.2)
t(5;17)(q32;p13)
t(5;10)(q32;q21)
t(3;5)(q25;q35)[b]

Data from Vardiman et al. [186]

[a]Defined as the presence of ≥3 unrelated abnormalities, none of which can be a balanced abnormality defining one of the entities within the WHO "AML with myelodysplasia-related changes" category

[b]Because this translocation can be often found in therapy-related AML, therapy-related disease should be excluded first for this translocation to support the diagnosis of AML with myelodysplasia-related changes

Translocation (8;21) is among the most frequent chromosomal aberrations in AML, occurring in 5–6 % of adults (Fig. 13.2 and data from reference no. [17]) and 12–14 % of children with AML [17, 25]. Much less often, cytogenetic variants of t(8;21) are encountered; these include recurrent insertions, i.e., ins(8;21) and ins(21;8), and three-way translocations, e.g., t(8;10;21), t(8;12;21), t(8;17;21) or t(8;20;21) (Table 13.1). Although the t(8;21) represents a primary chromosome

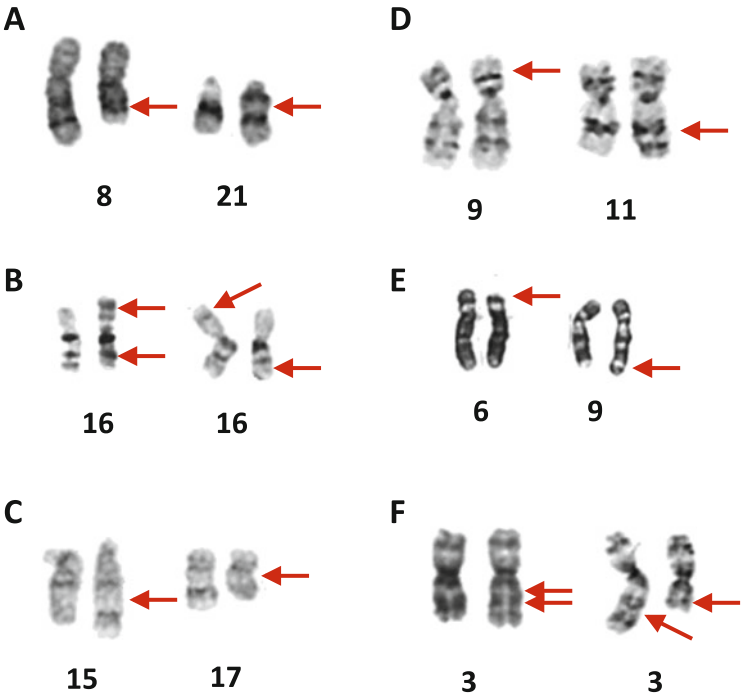

Fig. 13.1 G-banded partial karyotypes showing chromosome abnormalities used to denote specific entities in adults with AML within the WHO category entitled "AML with recurrent genetic abnormalities". (**a**) t(8;21)(q22;q22). (**b**) inv(16)(p13.1q22) (*left*) and t(16;16)(p13.1;q22) (*right*). (**c**) t(15;17)(q22;q12). (**d**) t(9;11)(p22;q23). (**e**) t(6;9)(p23;q34). (**f**) inv(3)(q21q26.2) (*left*) and t(3;3)(q21;q26.2) (*right*). *Arrows* indicate breakpoints in the rearranged chromosomes

aberration, it is detected as the sole cytogenetic abnormality in only ~30 % of patients. Among secondary chromosome abnormalities, the most frequent by far is −Y, found in roughly 60 % of male patients with t(8;21), followed by −X, seen in 33–40 % of female patients, and by del(9q) (17 %), +8 (5–7 %), and +4 (4 %) [52, 53]. The molecular consequences of these secondary aberrations are not yet well understood, although there are data indicating that del(9q) may act through loss of *TLE1* and *TLE4*, the putative tumour suppressor genes mapped to 9q21.3 [58]. Clearly, additional genetic rearrangements cooperating with *RUNX1-RUNX1T1* are required because the presence of *RUNX1-RUNX1T1* alone has been shown to be insufficient to induce leukaemia [59]. Recent studies revealed that such cooperating alterations include mutations in the *KIT* gene (detected in around 25 % of patients), *NRAS* and *KRAS* mutations (10–20 %), internal tandem duplications of the *FLT3* gene (*FLT3*-ITD; 7 %) and mutations in the *FLT3* tyrosine kinase domain (*FLT3*-TKD; 4 %) [59].

With regard to morphology, the presence of the t(8;21)/*RUNX1-RUNX1T1* is strongly, albeit not entirely, associated with AML with maturation in the neutrophil lineage. An increased number of eosinophil precursors, but without abnormalities

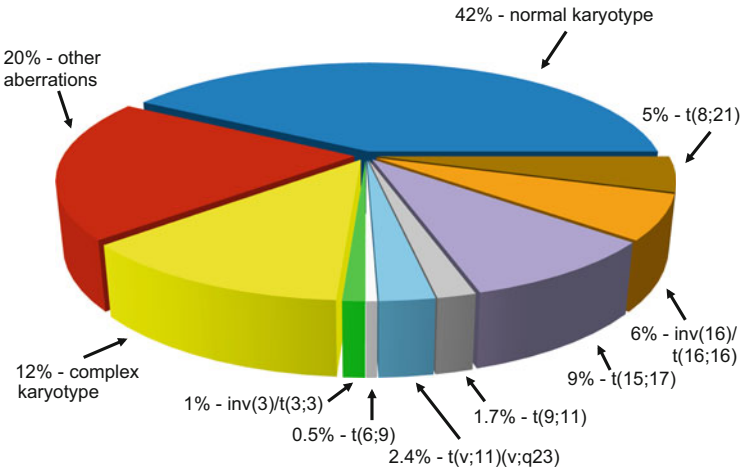

Fig. 13.2 Frequency distribution of patients harbouring chromosome abnormalities among 4,246 adults with AML enrolled onto CALGB companion protocol 8461. "Complex karyotype" denotes the presence of three or more abnormalities other than t(8;21), inv(16) or t(16;16), t(15;17), t(9;11), t(v;11)(v;q23), t(6;9), or inv(3) or t(3;3). "t(v;11)(v;q23)" denotes balanced rearrangements involving band 11q23 other than t(9;11)(p22;q23)

encountered in AML with inv(16), and distinctive pink-colored cytoplasm of neutrophils appear to differentiate t(8;21)-positive patients from other patients with AML with maturation who do not carry this rearrangement [60]. Detection of the t(8;21)/*RUNX1-RUNX1T1* is sufficient to make a diagnosis of AML, even if the overall percentage of marrow blasts is lower than 20 % [10].

Patients with the t(8;21)/*RUNX1-RUNX1T1* have a relatively favourable prognosis [14–17, 52, 53], especially when repetitive cycles of high-dose cytarabine are administered as postremission therapy [61]. While the clinical outcome does not seem to be affected by secondary chromosome aberrations, *KIT* mutations have been repeatedly shown to constitute an adverse prognostic factor [59, 62]. A recent study found that high cumulative incidence of relapse (CIR), but not shorter OS, was associated only with higher relative *KIT* mutant levels, i.e., when a percentage of mutated *KIT* alleles was ≥25 % of total *KIT* alleles [63]. Likewise, shorter OS, but not higher CIR, were found associated with high levels of *FLT3*-ITD [63]. These results await corroboration.

13.3.2 AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22)/CBFB-MYH11

The second CBF-AML entity is characterized by the presence of inv(16)(p13.1q22), or, less frequently, t(16;16)(p13.1;q22), which together are found in 5–6 % of adult (Fig. 13.2 and data from reference no. [17]) and 6–7 % of paediatric AML patients [17, 25]. At the molecular level, both these chromosome rearrangements fuse *CBFB*

with *MYH11*, a gene mapped to 16p13.1. The chimeric CBFB-MYH11 protein retains the ability to interact with *RUNX1* and block CBF dependent transcription. The genomic breakpoints within *CBFB* and *MYH11* are quite variable and, consequently, more than ten differently sized *CBFB-MYH11* fusion transcript variants have been reported [64]. The most common fusion type A is found in 85 % of patients, followed by type D and type E fusions, each detected in 5–10 % of patients. Although biologic and prognostic implications of different fusions are still unclear, our recent study revealed striking differences in the frequency of secondary chromosome aberrations and *KIT* mutations between patients with type A fusions compared with patients carrying non-type A fusions. Specifically, the latter harboured significantly more often +8 and +21 but less often, +22, which was detected exclusively in patients with type A fusions, as were prognostically unfavourable *KIT* mutations [64].

The presence of the inv(16)/t(16;16)/*CBFB-MYH11* is highly correlated with myelomonocytic marrow morphology and abnormal eosinophils, which constitute a pathognomonic feature of this AML type. The abnormal eosinophils are almost always present, although they may be very rare, comprising no more than as 0.2 % of marrow cells. As in the case of the t(8;21)/*RUNX1-RUNX1T1*, patients with the inv(16)/t(16;16)/*CBFB-MYH11* and marrow blasts percentages <20 % are diagnosed with AML [10].

Patients with the inv(16)/t(16;16)/*CBFB-MYH11* have a relatively favourable prognosis, particularly if their postremission treatment includes three to four cycles of high-dose cytarabine [65]. Among the recurrent secondary chromosome abnormalities, only the presence of +22 has been repeatedly found to reduce the patients' risk of relapse [52, 53, 66], and lengthen their OS duration [22, 66]. On the other hand, mutations in *KIT* [62, 66] and *FLT3* mutations, predominantly *FLT3*-TKD [59, 66, 67], adversely influence the patients' OS. Mutations in *NRAS* and *KRAS* are frequent (acquired by over 50 % of patients), but have not been found to constitute a prognostic factor [63, 66, 67]. However, their presence appears to render AML blasts more sensitive to higher doses of cytarabine given as part of postremission treatment [68].

13.3.3 AML with t(15;17)(q22;q12); PML-RARA

The third category of "AML with recurrent genetic abnormalities" in the WHO classification is acute promyelocytic leukaemia (APL), which constitutes 8–9 % of adult (Fig. 13.2 and data from reference no. [17]) and up to 10 % of childhood AML cases [17, 25]. The underlying molecular event in APL leukaemogenesis is creation of the gene fusion between the *RARA* gene, encoding the retinoic acid receptor α, and one of a number of partner genes, among which *PML* is by far the most frequent (98–99 %). In the majority of patients, the *PML-RARA* fusion gene is generated by a reciprocal translocation t(15;17) or its three- or four-way variants that involve one

or two chromosomes in addition to chromosomes 15 and 17. However, in ~4 % of APL patients, the karyotype may be normal because the *PML-RARA* fusion is formed by an insertion of a tiny segment containing the *RARA* gene into the *PML* locus [69]. Such cryptic rearrangements can be identified only by using reverse transcription-polymerase chain reaction (RT-PCR), fluorescence in situ hybridization (FISH), or next generation mRNA or genome sequencing, but neither marrow morphology nor clinical characteristics and response to treatment differ between patients with the standard t(15;17) and those with hidden alterations identifiable exclusively by molecular techniques [70].

In less than 1 % of APL patients, genes other than *PML* are fused with *RARA* as a result of variant rearrangements, mostly translocations. These variant *RARA* rearrangements and the resulting gene fusions include four reported recurrently, i.e., t(11;17)(q23;q21) and *ZBTB16-RARA*; t(4;17)(q12;q21) and *FIP1L1-RARA*; t(5;17)(q35;q21) and *NPM1-RARA*; and the microscopically undetectable *STAT5B-RARA* fusion [5, 71]. An additional five rearrangements were discovered thus far in single patients, viz, t(11;17)(q13;q21) and *NUMA1-RARA* [72]; t(X;17)(p11;q21) and *BCOR-RARA* [73]; der(2)t(2;17)(q32;q21) and *NABP1-RARA* [74]; t(3;17)(q26;q21) and *TBL1XR1-RARA* [75]; and a complex rearrangement within chromosome 17q involving an insertion of the *RARA* gene into a locus distal to the *PRKAR1A* gene at 17q24.2 and subsequent deletion creating the *PRKAR1A-RARA* fusion [76].

Ascertainment of the type of the fusion gene is important because it can determine whether the patient will respond to targeted therapy using all-*trans*-retinoic acid (ATRA) and arsenic trioxide (ATO). Therapeutic doses of ATRA have been shown to be effective in patients with the classic *PML-RARA* fusion as well as in those with variant fusions between *RARA* and the *FIP1L1*, *NPM1*, *NUMA1*, *BCOR* and *NABP1* genes [73, 74, 77]. In contrast, APL variants with *ZBTB16-RARA* and *STAT5B-RARA* fusions are resistant to ATRA, and are associated with a worse prognosis. Moreover, only APL with *PML-RARA* has been hitherto responsive to treatment with ATO [77].

Translocation (15;17)/*PML-RARA* and its variants are strongly correlated with distinct marrow morphology, in which abnormal promyelocytes dominate. Two major morphologic subtypes of APL are recognized, namely hypergranular (or typical) present in ~75 % of cases and microgranular (or hypogranular). The latter can sometimes be misdiagnosed morphologically as acute monocytic leukaemia and is associated with very high leukocyte counts with abundant abnormal microgranular promyelocytes, and the presence of the *FLT3*-ITD [10, 78]. Similarly to CBF-AML, patients with the t(15;17)/*PML-RARA* are diagnosed with APL regardless of marrow blast percentage [10].

The prognosis of APL patients with the t(15;17)/*PML-RARA*, which historically had been one of the worst among subtypes of AML, has become the most favourable with the use of treatment regimens containing ATRA and/or ATO, with CR rates of 90–95 % and a cure rate of up to 85 % in recent studies [79]. Secondary abnormalities, which accompany the t(15;17) in roughly one-third of APL patients

at diagnosis and include +8 or trisomy of 8q most frequently, do not seem to affect the patients' prognosis [22, 80]. However, in a recent study patients with a complex karyotype, i.e., two or more aberrations in addition to the t(15;17), had a significantly lower CR rate and shorter OS than patients with non-complex karyotype [81]. Since almost one-half of patients with a complex karyotype had aberrations involving chromosome 17, which mostly led to the loss of 17p and the *TP53* locus, the role of potential loss and/or mutations of *TP53* should be examined in APL with a complex karyotype [82]. *FLT3*-ITD, which is an established adverse prognostic factor in cytogenetically normal AML (CN-AML), has been associated with an increased incidence of induction death among adults [83] and children [84] with APL, but none of the large studies demonstrated significant differences in CR rates [83, 85], risk of relapse [83, 85], CIR [78], DFS [85], event-free survival (EFS) [86], or OS [83, 86] between patients with and without *FLT3*-ITD. However, the presence of both *FLT3*-ITD [78] and secondary chromosome abnormalities [87] was associated with shorter survival among APL patients who experienced a relapse.

13.3.4 AML with t(9;11)(p22;q23); KMT2A(MLL)-MLLT3

Translocation (9;11)(p22;q23) is the most frequent among over 120 chromosome abnormalities involving chromosome band 11q23 and the *KMT2A(MLL)* gene [88]. The *KMT2A(MLL)* gene encodes a DNA-binding protein methylating histone H3 lysine 4 (H3K4), and positively regulating expression of multiple genes including the *HOX* genes [89]. The translocation occurs in ~2 % of adults with AML (Fig. 13.2 and data from reference no. [17]) and results in a fusion of the *KMT2A(MLL)* gene with *MLLT3*, a gene residing at band 9p22 and encoding a nuclear protein containing serine-rich and proline-rich regions, which appear to be important for leukaemogenesis. Almost two-thirds of the cases carry t(9;11) as an isolated chromosome abnormality; +8 is the most frequent secondary aberration, seen in ~20 % of patients; followed by secondary +19 and +21. Morphologically, t(9;11) is strongly associated with acute monocytic and myelomonocytic leukaemias [10].

In most [16, 22, 90–92], but not all [25, 93], studies, patients with the t(9;11) had better clinical outcome that patients harbouring other rearrangements involving 11q23/*KMT2A(MLL)*, referred to in the WHO classification as "variant *MLL* translocations" [10], the recurrent of which are listed in Table 13.1. Consequently, t(9;11) has been classified in the intermediate cytogenetic-risk category [16, 22], whereas the variant *KMT2A(MLL)* translocations, which altogether comprise ~2 % of adults with AML (Fig. 13.2), belong to the unfavourable prognostic group [16, 22]. Interestingly, in a recent large paediatric series [94], +19 occurring as an abnormality secondary to t(9;11) was an independent adverse prognostic factor for incidence of relapse, EFS and OS. In contrast, t(9;11)-positive patients with a secondary +8 had a significantly lower incidence of relapse than children without +8 [94]. These results require corroboration.

13.3.5 AML with t(6;9)(p23;q34); DEK-NUP214

A recurrent t(6;9)(p23;q34) that creates the fusion gene *DEK-NUP214* is relatively rare, being detected in 0.5–0.7 % of adult (Fig. 13.2 and data from reference no. [55]) and 1.4–1.7 % of childhood patients with AML [55, 95]. Secondary cytogenetic abnormalities are uncommon, and ~90 % of the cases harbour t(6;9) or its rare three-way variants as the only chromosome change. On the other hand, the incidence of *FLT3*-ITD in patients with t(6;9) is the highest among AML cytogenetic subtypes, with 67–73 % of t(6;9)-positive patients carrying this mutation [55, 95].

Frequent morphologic features in adults with t(6;9) include increased basophilia (≥2 %), which is otherwise rare in AML, and single or multilineage dysplasia in the marrow [55].

The clinical outcome of patients treated with chemotherapy is very poor both in adults and children [55, 95], and does not seem to be associated with the presence or absence of *FLT3*-ITD [95]. However, the patients' prognosis can be considerably improved by allogeneic stem cell transplantation (SCT), especially in patients who are in CR at the time of transplantation [96]. Moreover, the DEK-NUP214 fusion protein was recently shown to increase cellular proliferation through upregulation of the signal transduction protein mTOR, thus indicating that t(6;9)-positive patients might benefit from treatment with mTOR inhibitors [97].

13.3.6 AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1(MECOM)

Both inv(3)(q21q26.2), and its less frequent variant t(3;3)(q21;q26.2), which in the past was occasionally described as ins(3;3)(q26;q21q26), lead to the fusion of the *RPN1* gene with a gene encoding the zinc finger transcription factor EVI1, currently re-named *MECOM* [31], that is involved in normal haematopoiesis and overexpressed as a result of inv(3) and t(3;3). These chromosome abnormalities are together detected in 1–1.4 % of patients with AML (Fig. 13.2 and data from reference no. [51]), both de novo and secondary to antecedent MDS, and are associated with multilineage dysplasia, abnormal megakaryopoiesis with micromegakaryocytes in the marrow and either normal or increased platelet counts and higher white blood cell counts at diagnosis [51, 98]. The majority of patients carry secondary aberrations in addition to inv(3) and t(3;3), of which monosomy 7 is the most frequent being present in one-half of all patients. In fact, the high incidence of −7 coexisting with inv(3), which in itself is a subtle rearrangement and may be occasionally missed by a cytogenetic laboratory [27], has alerted cytogeneticists to always examine chromosome 3 homologs closely in patients with a seemingly sole −7.

The clinical outcome of patients with inv(3) and t(3;3) has been repeatedly found to be very poor irrespective of the presence of −7 [16, 22], although in one study [51], patients with −7 fared even worse than those who did not harbour this monosomy. At present, allogeneic SCT appears to be the only therapeutic option capable of improving the dismal prognosis of inv(3) or t(3;3)-positive patients [99].

13.3.7 AML with t(1;22)(p13;q13); RBM15-MKL1

In contrast to the aforementioned subtypes of AML, AML with the t(1;22) occurs exclusively in children, 80 % of whom are younger than 1 year, and is very rare, with less than 50 cases reported worldwide to date [5]. The majority of patients have the t(1;22) as the sole chromosome alteration, but complex, hyperdiploid karyotypes are detected in ~40 % of the patients [39]. Strikingly, most infants below the age of 6 months carried the t(1;22) alone, whereas in over 80 % of older children the t(1;22) was part of a complex karyotype [39]. Essentially all patients present with acute megakaryoblastic leukaemia with extramedullary involvement, most often hepatosplenomegaly. While the prognosis was described initially as poor, albeit with a few long term survivors [39], Duchayne et al. [100] reported that those t(1;22)-positive children who did not die early responded well to intensive AML chemotherapy and/or SCT and had prolonged DFS and OS.

13.4 AML with Myelodysplasia-Related Changes

For patients without any of the recurrent genetic abnormalities described above, the WHO classification uses the presence of further specific cytogenetic findings to place some of them in another AML category called “AML with myelodysplasia-related changes”. To be assigned to this AML subtype, the patient with ≥20 % of bone marrow or blood blasts should fulfil one of three criteria: (i) disease progression from an antecedent MDS or myelodysplastic/myeloproliferative neoplasm; (ii) morphologic evidence of multilineage dysplasia in the marrow; or (iii) the presence of one of nine specific reciprocal translocations or eight unbalanced abnormalities and/or a complex karyotype with ≥3 unrelated chromosome changes (Table 13.2) [10]. Notably, fulfilment of the cytogenetic criterion is sufficient for diagnosis even in the absence of the other two criteria. With the exception of the t(3;5)(q25;q35)/*NPM1-MLF1*, which in the past was also described as t(3;5)(q21;q31), and is detected in ~0.4 % of AML patients and associated with an intermediate prognosis [22], the remaining reciprocal translocations are very rare, as is an unbalanced idic(X)(q13), which makes assessment of their clinical significance difficult [101]. Other unbalanced aberrations, save for del(11q) and del(9q), occur more often than not as part of a complex karyotype and are associated with poor outcome. However, the prognostic significance of −5 outside of a complex

karyotype is essentially unknown because it is very rare in patients with non-complex karyotypes. Additionally, patients with a complex karyotype with monosomy 5 ascertained by standard cytogenetic analysis also do not harbour true −5, since almost invariably parts from an apparently missing chromosome 5 are discovered using spectral karyotyping (SKY) [102] or FISH [103] in marker chromosomes and/or unbalanced structural aberrations that are partially recognized in G-banded preparations. Preliminary data indicate that patients with del(9q) differ clinically from patients with other unbalanced abnormalities, which indicates that their inclusion in the WHO "AML with myelodysplasia-related changes" should be reconsidered [101].

13.5 Correlations Between Cytogenetic Findings and Clinical Outcome of AML Patients

Pretreatment cytogenetic findings have been repeatedly proven to constitute one of the most important, independent prognostic factors in AML. This was demonstrated for the first time in the early 1980s by the results of a large, prospective, multi-centre study conducted by the Fourth International Workshop on Chromosomes in Leukaemia [104]. Both subsequent follow-up Workshop studies [11, 105, 106] and several single-institution [12, 107, 108] and large, collaborative multi-institutional studies [13–16, 18–22, 24–26, 109] have confirmed that karyotype at diagnosis is an independent prognostic determinant for achievement of CR, DFS, relapse risk and OS. Consequently, both the National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology [110] and the European LeukemiaNet (ELN) [111] stipulate that cytogenetic analysis is an obligatory component of the diagnostic work-up of patients with AML.

A number of large collaborative studies proposed prognostic prioritization systems classifying AML patients into favourable, intermediate or adverse risk groups based on pretreatment cytogenetic findings (Table 13.3) [15, 16, 22]. These cytogenetic risk systems have many common aspects, but also differ with respect to certain features (Table 13.3). In the MRC classification, first proposed in 1998 [14] and revised in 2010 [22], all abnormalities that are not categorized as favourable or adverse, and do not occur together with additional chromosome alterations belonging to either the favourable or adverse group, are classified in the intermediate-risk group. In contrast, both the Cancer and Leukaemia Group B (CALGB; currently known as the Alliance for Clinical Trials in Oncology) [16] and Southwest Oncology Group/Eastern Cooperative Oncology Group (SWOG/ECOG) [15] classifications explicitly categorize particular abnormalities into risk groups, which means that aberrations not frequent enough for analysis remain outside of these prognostic schemata. In addition, SWOG/ECOG and MRC classify patients with a given abnormality into a risk group once [15, 22], whereas CALGB provides risk-group assignment separately for probability of attaining a CR, CIR and OS [16]. As a result, in the latter schema, patients with the same abnormality [e.g., t(6;11)

Table 13.3 Cytogenetic-risk categorization of adult patients with the more frequent chromosome abnormalities in the three major collaborative studies of AML

Abnormality	Cytogenetic risk group				
	CALGB[a]			SWOG/ECOG[b]	MRC[c]
	Probability of CR attainment	Cumulative incidence of relapse	Overall survival		
t(8;21)(q22;q22)	Favourable	Favourable	Favourable	Favourable[d]	Favourable
inv(16)(p13.1q22) or t(16;16)(p13.1;q22)	Favourable	Favourable	Favourable	Favourable	Favourable
t(15;17)(q22-24;q12-21)	NA	NA	NA	Favourable	Favourable
t(9;11)(p22;q23)	Intermediate	Intermediate	Intermediate	Adverse[e]	Intermediate
t(6;11)(q27;q23)	Intermediate	Not classified	Adverse	Adverse[e]	Adverse
t(11;19)(q23;p13.1)	Intermediate	Not classified	Adverse	Adverse[e]	Intermediate[f]
abn(11q23)	Not classified	Not classified	Not classified	Adverse[e]	Adverse
t(6;9)(p23;q34)	Intermediate	Not classified	Adverse	Adverse[e]	Intermediate
inv(3)(q21q26.2) or t(3;3)(q21;q26.2)	Adverse	Not classified	Adverse	Adverse[g]	Adverse
-7	Intermediate	Adverse	Adverse	Adverse	Adverse
del(7q)	Not classified	Not classified	Not classified	Adverse	Adverse
loss of 7q	Intermediate	Not classified	Intermediate	Unknown	Adverse
-5	Not classified	Not classified	Not classified	Adverse	Adverse
del(5q)	Intermediate	Not classified	Intermediate	Adverse	Adverse
loss of 5q	Not classified	Not classified	Not classified	Unknown	Adverse
abn(17p)	Not classified	Not classified	Not classified	Adverse	Adverse
del(11q)	Intermediate	Not classified	Intermediate	Adverse[e]	Intermediate
abn(12p)	Adverse	Not classified	Intermediate	Intermediate/ Unknown[h]	Intermediate
del(9q)	Intermediate	Intermediate	Favourable/ Intermediate	Adverse	Intermediate
complex karyotype ≥3 abnormalities	Adverse	Adverse	Adverse	Adverse	NA

(continued)

Table 13.3 (continued)

complex karyotype ≥4 abnormalities	NA	NA	NA	NA	Adverse[i]
complex karyotype ≥5 abnormalities	Adverse	Adverse	Adverse	Adverse	NA
None (i.e., a normal karyotype)	Intermediate	Intermediate	Intermediate	Intermediate	Intermediate
-Y	Intermediate	Intermediate	Intermediate	Intermediate	Intermediate
+6	Not classified	Not classified	Not classified	Intermediate	Intermediat
+8 sole	Intermediate	Intermediate	Adverse	Intermediate	Intermediate
+8 with 1 other abnormality	Intermediate[j]	Intermediate	Adverse	Intermediate	Intermediate[k]
+11	Intermediate	Intermediate	Intermediate	Unknown	Intermediate
+13	Intermediate	Intermediate	Intermediate	Unknown	Intermediate
+21	Intermediate	Adverse	Intermediate	Unknown	Intermediate
t(9;22)(q34;q11.2)	NA	NA	NA	Adverse	Adverse
abn(3q)	Not classified	Not classified	Not classified	Adverse	Adverse[l]
del(16q)	Not classified	Not classified	Not classified	Favourable	Intermediate
del(20q)	Intermediate	Not classified	Intermediate	Adverse[m]	Intermediate
abn(20q)	Not classified	Not classified	Not classified	Adverse	Intermediate
abn(21q)	Not classified	Not classified	Not classified	Adverse	Intermediate

abn abnormality, *CALGB* Cancer and Leukaemia Group B, *CR* complete remission, *MRC* United Kingdom Medical Research Council; *NA* not available, *SWOG/ECOG* Southwest Oncology Group/Eastern Cooperative Oncology Group

[a]Data from Byrd et al. [16] who analyzed 1,213 adults (age range, 15–86 years, median 52 years) with de novo AML, excluding patients with t(15;17) and t(9;22)

[b]Data from Slovak et al. [15] who analyzed 609 adults (age range, 16–55 years, median 39 years) with AML

[c]Data from Grimwade et al. [22] who analyzed 5,876 adults (age range, 16–59 years, median 44 years) with de novo or secondary AML

[d]Patients with t(8;21) are classified in the favourable category only if the karytoype is not complex (i.e., comprises one or two abnormalities) and does not contain del(9q). Both del(9q) and complex karyotype were classified in the adverse category

[e]Would be included in "abn 11q" category

[f]t(11;19)(q23;p13.1) was not distinguished from t(11;19)(q23;p13.3) and both were included in the "t(11;19)(q23;p13)" category in this study

[g]Would be included in "abn 3q" category

[h]del(12p) classified in the intermediate category, other abnormalities involving 12p seemingly classified as unknown

[i]After exclusion of patients with abnormalities conferring favourable or adverse prognosis

[j]Any abnormality other than t(8;21), inv(16), t(16;16) or t(9;11)

[k]The abnormality occurring together with +8 may not by classified in the favourable or adverse category

[l]Excluding t(3;5)(q21~25;q31~35)

[m]Would be included in "abn 20q" category

(q27;q23)] may be categorized in the intermediate-risk group concerning probability of induction success, in the adverse-risk group with regard to OS and not classified for CIR, because the number of patients who achieved a CR was too low for CIR risk-group assignment [16].

Nevertheless, in spite of the differences among classifications, many chromosome aberrations are uniformly assigned to either a favourable risk, e.g., t(15;17) and CBF-AML-related inv(16)/t(16;16) and t(8;21), intermediate risk, e.g., −Y, or adverse risk, e.g., inv(3) or t(3;3), −7 and a complex karyotype, categories. Notably, in contrast to CALGB and MRC [16, 22], the SWOG/ECOG classification included patients with del(16q) in the favourable risk-group [15]. However, we believe that unless such patients are shown to carry a misinterpreted inv(16) or t(16;16) and the resultant *CBFB-MYH11* gene fusion, they should not be included in the favourable risk group because del(16q) is typically detected in AML with morphology other than that of acute myelomonocytic leukaemia with abnormal eosinophils, and is not associated with a favourable outcome [112–114].

All classifications agree that complex karyotype is associated with unfavourable prognosis, but the definition of a complex karyotype differs among studies. While SWOG/ECOG [15], CALGB [16], and the German AML Study Group [115] considered as complex karyotypes those containing three or more abnormalities, a multi-centre Italian study defined complex karyotype as "the presence of a clone with more than three cytogenetic abnormalities" [109], and the initial MRC definition specified a complex category as "the presence of a clone with at least five unrelated cytogenetic abnormalities" [14]. In most instances, the definition of complex karyotype did not include patients with t(8;21), inv(16)/t(16;16), or t(15;17) following data showing that patients with these abnormalities constitute separate biological and clinical entities, in which increased karyotype complexity does not affect adversely clinical outcome in a manner comparable to other patients with ≥3 abnormalities [14, 16, 52, 53, 80]. In some studies, the complex karyotype category also excludes patients harbouring t(9;11)(p22;q23) [16, 19] or any balanced rearrangement involving band 11q23 [20]. The revised MRC classification defined complex karyotype as one with four or more abnormalities, which excluded all chromosome changes that themselves bestowed either a favourable prognosis [i.e., t(8;21), inv(16)/t(16;16), t(15;17)] or an unfavourable prognosis [i.e., abn(3q) other than t(3;5); inv(3))/t(3;3); add(5q), del(5q), −5; −7, add(7q)/del(7q); t(6;11), t(10;11)(p12;q23), other t(11q23) excluding t(9;11) and t(11;19)(q23;p13) (19p13.1 or 19p13.3 breakpoint was not specified); t(9;22)(q34;q11.2); −17/abn(17p)] [22]. Finally, the ELN specified complex karyotype as having ≥3 abnormalities in the absence of any balanced rearrangements used by the WHO Classification to denote "AML with recurrent genetic abnormalities" [111]. Because the latter two definitions of complex karyotype have been introduced recently, it is currently unknown whether one of them is better than the other.

Depending on the definition, AML patients with a complex karyotype comprise 10–12 % of all AML patients, if complex karyotype is defined as ≥3 aberrations [15, 16, 115], or 8–9 %, if ≥5 aberrations are necessary for complex karyotype recognition [14–16, 18]. In individual patients, complex karyotypes can comprise

variable numbers of chromosome aberrations that in rare cases can reach as many as 30, but the occurrence of particular structural and numerical abnormalities is not random [116]. Balanced rearrangements (translocations, insertions, or inversions) are relatively rare, and unbalanced aberrations leading to loss of chromosome material (monosomies, deletions, and unbalanced translocations) predominate. Most frequently lost is chromosome material from, in decreasing order, chromosome arms 5q, 17p, 7q, 18q, 16q, 17q, 12p, 20q, 18p, and 3p. Recurrent gains of chromosomal segments are less frequent and are often hidden in marker chromosomes and unbalanced translocations; they most often involve 8q, 11q, 21q, 22q, 1p, 9p, and 13q [116]. In ~80 % of patients with complex karyotype loss of 5q material is detected, whereas in ~50 % of the patients, parts of 7q and 17p are lost. Furthermore, abnormalities of 5q, 7q and 17p often occur together, in the same patient, which means that ~85 % of all patients with a complex karyotype harbour loss of at least one of these chromosome arms [116]. On the other hand, ~5 % of complex karyotype patients have only numerical abnormalities, mostly gains of chromosomes (most often +8, +13, +21, +14, +10, and +19) [117]. Chilton et al. [117] reported recently that OS of AML patients with a complex hyperdiploid karyotype (i.e., 49–65 chromosomes) that included only numerical abnormalities was significantly better than OS of patients with a hyperdiploid complex karyotype that had at least one abnormality associated with an adverse outcome [−5/del(5q), −7/del(7q), t(9;22), *KMT2A(MLL)* translocations except t(11;19)(q23;p13), or abnormalities of 3q or 17p]. Indeed, OS of the former was comparable to OS of patients classified in the MRC intermediate cytogenetic-risk category suggesting that patients with hyperdiploid complex karyotype with numerical abnormalities only should not be considered to have an adverse prognosis [117].

In 2008, Breems et al. [118] proposed recognition of a cytogenetic subset of patients with a particularly adverse outcome named monosomal karyotype (MK). The MK category excludes patients with CBF-AML and APL, and comprises AML patients who harbour two or more autosomal monosomies (i.e., loss of any chromosome but −Y or −X) or have one autosomal monosomy together with at least one structural chromosome abnormality (apparently excluding marker or ring chromosomes) [118]. Thus, MKs are very heterogeneous cytogenetically and frequently include abnormalities that have themselves been independently associated with adverse risk, including inv(3) or t(3;3), rearrangements involving 11q23/*KMT2A(MLL)*, or del(5q). Furthermore, up to 75 % of complex karyotypes are hypodiploid (i.e., contain ≤45 chromosomes, with ≥1 monosomy), and not infrequently ≥1 monosomy can be also found in the remaining complex karyotypes with a pseudodiploid (i.e., with 46 chromosomes) or hyperdiploid (i.e., with ≥47 chromosomes) modal chromosome numbers [116]. Consequently, most patients with a complex karyotype are also deemed to have a monosomal karyotype. While the MK designation has been useful in identifying AML patients with very poor outcomes [118–120], which could be improved to some extent by allogeneic SCT in first CR [121, 122], the marked heterogeneity of MKs makes it unlikely that a single or even a few molecular alterations that could be targeted therapeutically underlie the disease development in all MK patients. Moreover, inclusion of patients

into the MK category depends upon genetic methodology used to detect it. Following application of array-based comparative genomic hybridization and single-nucleotide polymorphism (SNP) genomic profiling, Rücker et al. [123] reduced greatly the number of MK cases from 78 % of all patients with a complex karyotype when MK was identified by standard karyotyping to only 32 %. This happened because many chromosomes deemed lost in G-banded karyotypes (monosomy) were in reality not totally lost, but their parts were found hidden in such structural abnormalities as marker chromosomes, ring chromosomes and unbalanced translocations with partially unidentified chromosome material [123].

Although the molecular consequences of the majority of losses and gains of specific chromosomes and/or chromosome segments in AML patients with complex karyotypes are not yet well characterized, the association between cytogenetic abnormalities of 17p, frequent in complex karyotypes, and mutations in and/or loss of the *TP53* gene causing loss of p53 protein function is well documented [123, 124]. *TP53* loss and/or mutations represent one of the molecular pathways responsible for marked genomic instability of complex karyotypes manifested by the simultaneous presence of multiple related clones and non-clonal cells, creation of complex abnormal chromosomes composed of material from three or more separate chromosomes and/or generation of abnormal "sandwich-like" chromosomes containing several small interchanging segments from two different chromosomes [116]. It has recently been shown that in up to one-half of AML cases with *TP53* mutations, a complex karyotype can arise through a single catastrophic event called chromothripsis, where numerous chromosome rearrangements are acquired simultaneously, instead of through a gradual, stepwise karyotype evolution [125]. Even though AML patients with a complex karyotype and *TP53* alterations have very poor outcome, with CR rates significantly lower and relapse-free survival, EFS and OS significantly shorter than those of patients without *TP53* alterations [123], the presence of chromothripsis has made the dismal prognosis of *TP53*-mutated patients even worse when compared with the outcome of *TP53*-mutated patients without evidence of chromothripsis [125].

The single largest cytogenetic subset of both adult and childhood AML comprises patients with an entirely normal karyotype, although the percentage of adults with CN-AML (40–45 %) is greater than that among paediatric cases (22–24 %). There are data suggesting that the proportion of patients with CN-AML established by standard cytogenetic analysis is overestimated because occasionally such subtle aberrations as t(11;19)(q23;p13.1), inv(3) or inv(16) may escape recognition in preparations of suboptimal quality. To minimize this possibility, CALGB/ALLIANCE pioneered and has been conducting successfully central karyotype review for the last 30 years [27]; central karyotype review is also performed by other cooperative groups [126]. Moreover, some CN-AML patients have been shown to harbour common AML-associated gene fusions, for instance *PML-RARA* or *CBFB-MYH11*, that are a result of microscopically undetectable rearrangements such as cryptic insertions, but these patients are rare and constitute only a fraction of all CN-AML cases [69, 114, 127].

Other cryptic rearrangements that are undetectable on routine cytogenetic investigation seem to be more frequent, but they occur mostly in childhood AML. These include a prognostically adverse t(5;11)(q35.2;p15.4)/*NUP98-NSD1*, detected in 16 % of paediatric and 2 % of adult CN-AML patients [36], and inv(16)(p13.3q24.3) resulting in the *CBFA2T3-GLIS2* gene fusion, which is a frequent rearrangement in childhood acute megakaryoblastic leukaemia and portends an inferior clinical outcome, but to date has not been detected in adults [128]. The latter inversion was initially reported in non-Down syndrome children with acute megakaryoblastic leukaemia, who in all but two cases had an abnormal, often complex, karyotype [128]. A subsequent study analyzed a cohort of 230 children with CN-AML, which did not include any patients with *KMT2A(MLL)*, *CBFB*, *NPM1*, or *FLT3* rearrangements, and detected inv(16)(p13.3q24.3)/*CBFA2T3-GLIS2* in 20 (8.4 %) patients, only one-half of whom was diagnosed with acute megakaryoblastic leukaemia [129]. Finally, a cryptic t(7;12)(q36.3;p13.2)/*MNX1-ETV6* has been occasionally reported in paediatric CN-AML, but to date a vast majority of cases with t(7;12) had an abnormal karyotype, which almost always contained trisomy of chromosome 19 [5]. This suggests that the presence of +19 in a child with AML warrants performing an RT-PCR or FISH analysis to confirm or refute the existence of t(7;12)(q36.3;p13.2)/*MNX1-ETV6*, an abnormality associated with an adverse prognosis [37].

As a group, patients with CN-AML have been classified in the intermediate prognostic category in all major cytogenetic-risk classifications, because their CR rates, DFS and OS were typically worse than those of adequately treated patients with the t(15;17), t(8;21) or inv(16), but better than the outcome of patients with adverse cytogenetic features [14–16, 22]. However, intensive research efforts undertaken during last the two decades have revealed that CN-AML is very heterogeneous at the molecular level, and the patients belong to molecular subsets with vastly varying prognoses [130]. Those molecular abnormalities that were reported to have an effect on clinical outcome of CN-AML patients are provided in Table 13.4. The presence of mutations in *NPM1* [131–135] and double mutations in *CEBPA* [136, 137] and high expression of *miR-181a* [138] have been associated with a favourable outcome, whereas the patients' prognosis is adversely affected by *FLT3*-ITD [139–142], *KMT2A(MLL)*-PTD [134, 143–145], mutations in *DNMT3A* (both R882 and non–R882 mutations) [146–148], *IDH1* [149, 150], *IDH2* (R172 mutations) [149, 150], *TET2* [151–153], *ASXL1* [154], *RUNX1* [155–157], *WT1* [158–160], and *BCOR* [161], expression of *GAS6* [162], and high expression of *BAALC* [163–166], *ERG* [167, 169], *MN1* [170–172], *SPARC* [173], *DNMT3B* [174], *miR-3151* [175], and *miR-155* [176] (details are provided in Table 13.4). Because leukaemic blasts of some CN-AML patients contain two or more (up to six) prognostic mutations and changes in gene expression, current research efforts concentrate on unravelling how combinations of multiple molecular genetic alterations influence the outcome of CN-AML patients.

Meanwhile, an international expert panel working on behalf of the ELN has recently incorporated three molecular genetic markers whose prognostic significance in CN-AML is best documented, namely *FLT3*-ITD, and *NPM1* and *CEBPA*

Table 13.4 Molecular genetic alterations influencing prognosis of patients with cytogenetically normal acute myeloid leukaemia (CN-AML)

Genetic alteration	Frequency	Associations with clinical outcome
Gene mutations		
Mutations in *NPM1*	46–62 %	Adults younger than 60 years with *NPM1* mutations in the absence of *FLT3*-ITD have significantly better CR rates, EFS, DFS, and OS than patients with wild-type *NPM1* [131–134]
		In patients aged 60 years or older, *NPM1* mutations alone constitute an independent favourable prognostic factor [135]
Double mutations in *CEBPA*	6–8 %	OS of patients with double *CEBPA* mutations was longer than OS of patients with wild-type *CEBPA* and of those with single *CEBPA* mutations. On MVA, double *CEBPA* mutations were an independent favourable factor for OS [136]
		In younger (<60 years) patients, both single and double *CEBPA* mutations conferred longer OS than OS of patients with wild-type *CEBPA*, but only double *CEBPA* mutations retained favourable prognostic significance in MVA for OS, EFS and RFS [137]
FLT3-ITD	28–39 %	Patients with *FLT3*-ITD have significantly shorter CRD, DFS and OS (but not CR rates) than patients who do not harbour *FLT3*-ITD [139, 140]
		Patients with high *FLT3* mutant to *FLT3* wild-type allele ratio have particularly poor prognosis [139, 141, 142]
KMT2A(MLL)-PTD	5–11 %	*KMT2A(MLL)*-PTD had no prognostic significance in intensively treated younger (<60 years) [187], and older (aged ≥60 years) patients [188], but in earlier studies patients with *KMT2A(MLL)*-PTD had shorter CR duration (but not CR rates or OS) [134, 143–145]
Mutations in *DNMT3A*	27–35 % in younger and 33 % in older patients	Overall, *DNMT3A* mutations are associated with shorter DFS (but not OS [147]), with lower CR rates and shorter OS on MVA [146], and shorter EFS and OS, which remained significant on MVA
		In patients aged <60 years, non-R882-*DNMT3A* mutations are associated with shorter DFS and OS [147]. In patients aged ≥60 years, R882-*DNMT3A* mutations confer shorter DFS and OS [147]
Mutations in *IDH1*	13–16 %	Overall, *IDH1* mutations are not associated with outcome [149, 150]
		In patients with *NPM1* mutations and no *FLT3*-ITD, *IDH1* mutations are associated with a higher relapse risk, shorter OS [150] and DFS [149]

(continued)

Table 13.4 (continued)

Mutations in *IDH2*	11–19 %	R172-*IDH2* mutations are associated with lower CR rates [149, 150], increased relapse risk and shorter OS [150]. R140-*IDH2* mutations are not associated with outcome [149]
Mutations in *TET2*	16–30 % overall; 15 % in younger and 29 % in older patients	*TET2* mutations are associated with lower CR rates [151] and shorter DFS [151], OS [151] and EFS [151, 152] and higher RR [152] in CN-AML patients classified in the ELN Favourable Genetic Group. Among patients with *NPM1* mutations but no *FLT3*-ITD, *TET2* mutations confer shorter EFS [152], higher RR [152], and shorter OS [153]
Mutations in *ASXL1*	5–10 % overall; 3 % in younger and 16 % in older patients	In patients aged ≥ 60 years, mostly those in the ELN Favourable Group, *ASXL1* mutations are associated with lower CR attainment probability and shorter DFS, OS and EFS [154]
Mutations in *RUNX1*	13–26 % overall; 6–8 % in younger and 16 % in older patients	*RUNX1*-mutated patients had lower CR rates and worse DFS, EFS and OS than patients with wild-type *RUNX1* [155–157]
Mutations in *WT1*	8–10 % overall; 11–13 % in younger and 7 % in older patients	*WT1* mutations confer worse DFS and OS (but not CR probability) [158]; worse CR achievement probability, CIR, DFS and OS [159, 160]; no impact on RFS or OS [189]
		Patients with *WT1* mutations and *FLT3*-ITD have lower CR rates and worse RFS and OS than patients with *WT1* mutations without *FLT3*-ITD [189]
Mutations in *BCOR*	4 %	*BCOR* mutations are associated with a shorter OS and EFS [161]

Genetic alteration	High vs. low expression	Associations with clinical outcome
Changes in gene expression		
High expression of *BAALC*	Median cut [163–166]	Younger (aged <60 years) high *BAALC* expressers have lower CR rates [164, 165], higher CIR [164] and inferior DFS [163], EFS [163] and OS [163, 165]. *BAALC* expression was confirmed as an independent risk factor on MVA [163–165]
		Older patients (aged ≥60 years) with high *BAALC* expression have lower CR rates, and shorter DFS and OS [166]
High expression of *ERG*	3 quartiles with lower expression vs. the 4th quartile [167, 169]	In younger patients (aged <60 years), high *ERG* expression is associated with lower CR rates [168] and worse CIR [167], EFS [168] and OS [167, 169]
	Median cut [166, 168]	In older patients (aged ≥60 years), high *ERG* expression is associated with shorter DFS [166] and OS [166, 169]

(continued)

Table 13.4 (continued)

High expression of *MN1*	Median cut [170, 172]	In younger patients (aged <60 years), high *MN1* expression (defined using median cut) is associated with shorter RFS and OS and higher RR [170]. When *MN1* expression was used as a continuous variable, higher *MN1* expression was associated with lower CR rates and shorter DFS and OS [171]
	Continuous variable [171]	
		In older patients (aged ≥60 years), high *MN1* expressers had lower CR rates and shorter EFS and OS [172]
High expression of *SPARC*	Median cut [173]	In younger patients (aged <60 years), high *SPARC* expression bestowed lower odds of achieving a CR and shorter OS (which remained significant on MVA) and shorter DFS (not significant on MVA) [173]
Expression of *GAS6*	*GAS6* expression vs. no detectable expression [162]	Patients who expressed *GAS6*, especially those aged ≥60 years, more often failed to achieve a CR and had shorter DFS and OS than patients without *GAS6* expression; all of which remained significant on MVA [162]
High expression of *DNMT3B*	Median cut [174]	Older patients (aged ≥60 years) with high *DNMT3B* expression had fewer CRs and shorter DFS and OS; ($P < 0.001$), which remained significant on MVA [174]
High expression of *miR-181a*	Continuous variable [138]	Younger patients (aged <60 years) with higher *miR-181a* expression have a higher CR rate and longer OS than patients with lower expression [138]
High expression of *miR-3151*	Median cut [175]	Older patients (aged ≥60 years) with high *miR-3151* expression have shorter DFS and OS, than patients with low expression [175]
High expression of *miR-155*	Median cut [176]	Patients with high *miR-155* expression have a lower CR rate and shorter DFS and OS than patients with low expression [176]

CIR cumulative incidence of relapse, *CR* complete remission, *CRD* CR duration, *DFS* disease-free survival, *EFS* event-free survival, *ELN* European LeukemiaNet, *FLT3*-ITD internal tandem duplication of the *FLT3* gene, *KMT2A(MLL)*-PTD partial tandem duplication of the *KMT2A(MLL)* gene, *MVA* multivariable analysis, *OS* overall survival, *RFS* relapse-free survival, *RR* risk of relapse, *vs*. versus

mutations, into a proposed standardized system for reporting cytogenetic and molecular abnormalities in studies correlating genetic findings with treatment outcome in AML [111]. These molecular markers are used to classify CN-AML patients into either the ELN Favourable Genetic Group, which also comprises patients with an abnormal karyotype with either the t(8;21)/*RUNX1-RUNX1T1* or inv(16)/t(16;16)/*CBFB-MYH11*, or into the Intermediate-I Group, which includes exclusively CN-AML patients who belong to one of three Genetic Subsets differing with regard to combinations of *NPM1*, *CEBPA* and *FLT3* mutational status.

The remaining Intermediate-II and Adverse Genetic Groups encompass patients with cytogenetic abnormalities only (for details please see Table 13.5) [111]. The ability of the four ELN Genetic Groups to predict treatment outcome has been recently examined by two large studies, each analyzing over 1,500 AML patients [177, 178]. Both studies yielded similar results showing that application of the ELN reporting system results in a prognostic separation of the favourable and adverse groups from each other and from both Intermediate Genetic Groups for all analyzed outcome endpoints, namely CR rates [178], DFS [178], probability of relapse [177] and OS [177, 178]. By performing multivariable analyses, the CALGB study also demonstrated that the association of ELN Genetic Groups with clinical outcome was independent from other established prognostic factors [178]. Interestingly, both studies revealed a difference between younger and older patients concerning the Intermediate-I and Intermediate-II Groups, with older patients in both Groups having virtually identical outcomes, and younger patients classified in the Intermediate-II Group having a significantly longer OS than the Intermediate-I Group patients (Fig. 13.3). The reasons for superior outcome of younger, but not older, patients in the Intermediate-II as opposed to the Intermediate-I Group are not fully understood. They may be related to striking cytogenetic heterogeneity of the Intermediate-II Group, which, in addition to t(9;11), consists of numerous structural and numerical abnormalities not classified as favourable or adverse whose distribution likely differs between younger and older patients in a manner similar to the age-related differences in the distribution of both the ELN Genetic Groups (Fig. 13.4) and Genetic

Table 13.5 The ELN standardized reporting system for correlation of cytogenetic and molecular genetic data with clinical data in acute myeloid leukaemia [111]

Genetic group	Genetic subset
Favourable	t(8;21)(q22;q22); *RUNX1-RUNX1T1*
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
	Mutated *NPM1* without *FLT3*-ITD (normal karyotype)
	Mutated *CEBPA* (normal karyotype)
Intermediate-I	Mutated *NPM1* and *FLT3*-ITD (normal karyotype)
	Wild-type *NPM1* and *FLT3*-ITD (normal karyotype)
	Wild-type *NPM1* without *FLT3*-ITD (normal karyotype)
Intermediate-II	t(9;11)(p22;q23); *MLLT3-MLL*(*KMT2A*)
	Cytogenetic abnormalities not classified as favourable or adverse
Adverse	inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*(*MECOM*)
	t(6;9)(p23;q34); *DEK-NUP214*
	t(v;11)(v;q23); *MLL*(*KMT2A*) rearranged
	−5 or del(5q)
	−7
	abnl(17p)
	Complex karyotype[a]

[a]Complex karyotype is defined as at least three chromosome abnormalities, excluding any of the World Health Organization-designated recurring translocations or inversions, i.e., t(8;21), inv(16) or t(16;16), t(15;17), t(9;11), t(v;11)(v;q23), t(6;9), inv(3) or t(3;3)

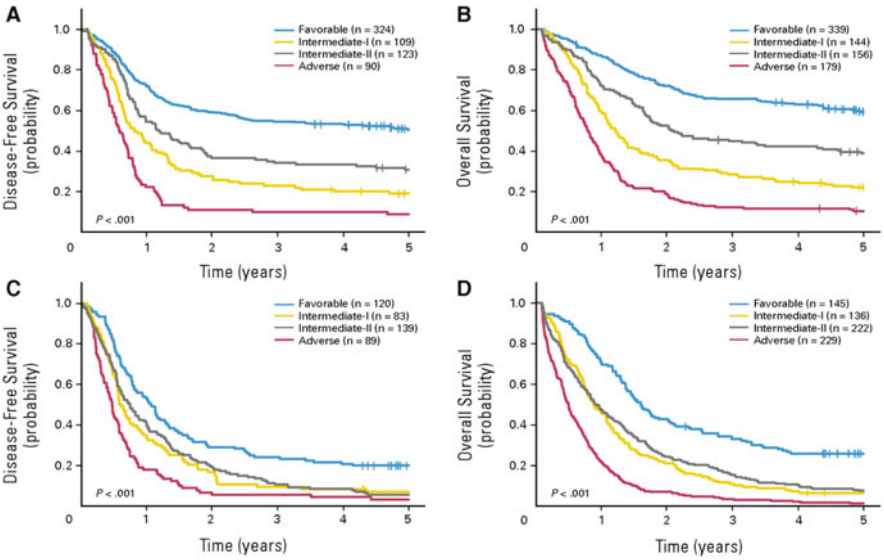

Fig. 13.3 Outcome of patients with de novo AML classified into the four European LeukemiaNet (ELN) Genetic Groups according to the ELN recommendations. (**a**) Disease-free survival and (**b**) overall survival of younger patients aged less than 60 years; (**c**) disease-free survival and (**d**) overall survival of older patients aged 60 years or older (Reprinted with permission from Mrózek et al. [178] © 2012 American Society of Clinical Oncology. All rights reserved)

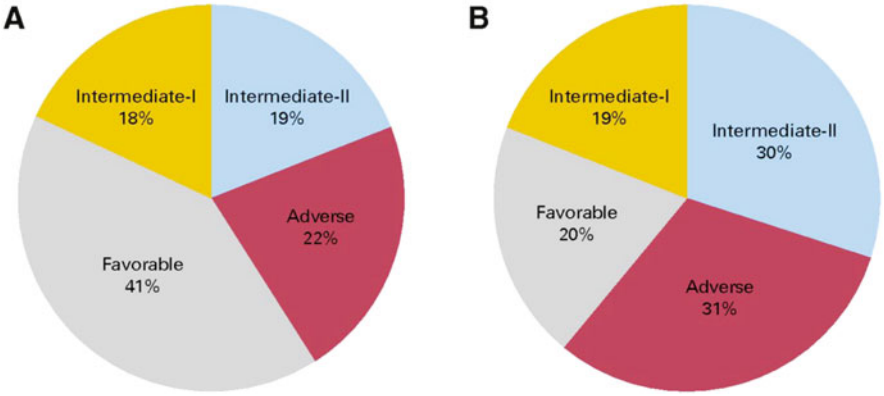

Fig. 13.4 Distribution of the ELN Genetic Groups in younger (**a**) and older (**b**) adults with de novo AML. The Favourable Group is significantly more ($P < .001$) and the Intermediate-II and Adverse Groups are less ($P < 001$) common among younger patients compared with older patients (Reprinted with permission from Mrózek et al. [178]. © 2012 American Society of Clinical Oncology. All rights reserved)

Subsets within these Groups (Fig. 13.5) [178]. These data support the view that the ELN classification should be applied to younger and older patients separately when used for risk stratification of AML patients in prospective clinical trials and in studies correlating genetic findings with clinical outcome [178]. Recent studies

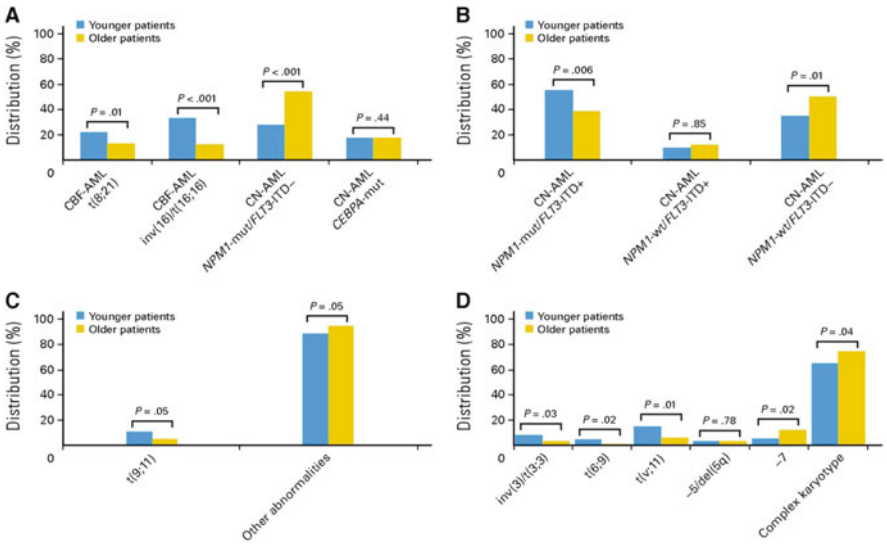

Fig. 13.5 Distribution of the genetic subsets within ELN Genetic Groups in younger and older adults with de novo AML. (**a**) The Favourable Group consists of four genetic subsets. The first two subsets are patients with core-binding factor AML with either t(8;21) or inv(16)/t(16;16). The second two subsets are patients with cytogenetically normal AML (CN-AML) with either *NPM1*-mut/*FLT3*-ITD – (i.e., mutated *NPM1* without *FLT3*-ITD) or *CEBPA*-mut (i.e., mutated *CEBPA*). (**b**) The Intermediate-I Group consists of three genetic subsets of patients with CN-AML and either *NPM1*-mut/*FLT3*-ITD+ (i.e., mutated *NPM1* and *FLT3*-ITD) or *NPM1*-wt/*FLT3*-ITD+ (i.e., wild-type *NPM1* and *FLT3*-ITD) or *NPM1*-wt/*FLT3*-ITD – (i.e., wild-type *NPM1* without *FLT3*-ITD). (**c**) The Intermediate-II Group consists of two genetic subsets of patients with either t(9;11) or other abnormalities (i.e., cytogenetic abnormalities not classified as favourable or adverse). (**d**) The Adverse Group consists of seven genetic subsets: (1) inv(3)/t(3;3), (2) t(6;9), (3) t(v;11) [i.e., various translocations involving 11q23/*KMT2A(MLL)* other than t(9;11)], (4) −5/del(5q) (i.e., monosomy of chromosome 5 or deletion of 5q), (5) −7 (i.e., monosomy of chromosome 7), (6) abnl(17p) (i.e., abnormalities of the short arm of chromosome 17; no patient had this abnormality in our study), or (7) a complex karyotype containing ≥3 cytogenetic abnormalities (Reprinted with permission from Mrózek et al. [178]. © 2012 American Society of Clinical Oncology. All rights reserved)

have provided evidence that testing for such genetic markers as mutations in the *TET2* [151], *ASXL1* [154], and *RUNX1* [156] genes and the expression levels of *MN1* [170], *miR-155* [176] and *miR-3151* [175] may refine the precision of patient risk stratification within the ELN Genetic Groups.

13.6 Concluding Remarks and Future Directions

During the last four decades, cytogenetic studies of AML have identified a large number of recurring chromosomal abnormalities with diagnostic and prognostic significance, and many of them have been dissected molecularly [3, 5, 17]. Cytogenetic analysis of pretreatment marrow has become a mandatory part of the

diagnostic work-up of patients suspected to suffer from AML. Pretreatment cytogenetic findings are being increasingly combined with the results of molecular genetic assays, as exemplified by the ELN reporting system [111], to guide the selection of the most effective treatment approaches. However, the prognostic significance of several recurrent but less frequent chromosomal abnormalities is yet to be established conclusively. They are often categorized in the intermediate-risk category (or the ELN Intermediate-II Genetic Group) by virtue of the absence of evidence that they confer a more favourable or adverse prognosis. Thus, further collaborative studies are needed to collect enough cases with these less common abnormalities to determine how they influence probability of CR achievement, DFS and OS. In addition, prognostic factors depend on the kind of therapy, which means that there is a continuing need for large prospective studies correlating cytogenetic and molecular genetic alterations with clinical outcome of both patients who are treated with contemporary regimens and of patients who are administered novel, experimental therapies often targeting specific genetic rearrangements.

This makes accurate detection of acquired genetic abnormalities of utmost importance. Recently, high-throughput next generation sequencing (NGS) technologies have been used to study AML genomes providing an unprecedented view of intricate interactions of genetic changes contributing to leukaemogenesis in individual patients [8, 179–181]. Although NGS technologies are being used successfully for research purposes they do not seem to be currently ready for routine use in diagnostics and prognostication of AML as stand-alone methods. This is due to the large amount of work and time necessary to corroborate results of these assays, their high, although rapidly declining, cost [179], as well as the need to fine tune bioinformatic algorithms in the programs analyzing sequencing data, which, as a recent study demonstrated [182], may occasionally fail to recognize a pathogenetically essential gene fusion created by a translocation recurrent in AML. It thus remains to be seen whether NGS technologies will be able to entirely supplant classical cytogenetics, FISH and RT-PCR in the future, or whether they will remain a powerful addition to the currently available armamentarium of techniques capable of detecting acquired genetic lesions with clinical significance in AML.

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References

1. Caspersson T, Zech L, Johansson C (1970) Differential binding of alkylating fluorochromes in human chromosomes. Exp Cell Res 60:315–319
2. Rowley JD (1973) Identification of a translocation with quinacrine fluorescence in a patient with acute leukemia. Ann Genet 16:109–112
3. Mrózek K, Heinonen K, Bloomfield CD (2001) Clinical importance of cytogenetics in acute myeloid leukaemia. Best Pract Res Clin Haematol 14:19–47
4. Walker A, Mrózek K, Kohlschmidt J, Rao KW, Pettenati MJ et al (2013) New recurrent balanced translocations in acute myeloid leukemia and myelodysplastic syndromes: Cancer and Leukemia Group B 8461. Genes Chromosomes Cancer 52:385–401

5. Mitelman F, Johansson B, Mertens F, (eds) Mitelman database of chromosome aberrations and gene fusions in cancer. Available at: http://cgap.nci.nih.gov/Chromosomes/Mitelman. Accessed 9 June 2014
6. Mitelman F, Johansson B, Mertens F (2007) The impact of translocations and gene fusions on cancer causation. Nat Rev Cancer 7:233–245
7. McCormack E, Bruserud O, Gjertsen BT (2008) Review: genetic models of acute myeloid leukaemia. Oncogene 27:3765–3779
8. Cancer Genome Atlas Research Network (2013) Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. N Engl J Med 368:2059–2074
9. Kelly L, Clark J, Gilliland DG (2002) Comprehensive genotypic analysis of leukemia: clinical and therapeutic implications. Curr Opin Oncol 14:10–18
10. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA et al (eds) (2008) WHO classification of tumours of haematopoietic and lymphoid tissues. IARC, Lyon
11. Arthur DC, Berger R, Golomb HM, Swansbury GJ, Reeves BR et al (1989) The clinical significance of karyotype in acute myelogenous leukemia. Cancer Genet Cytogenet 40:203–216
12. Schiffer CA, Lee EJ, Tomiyasu T, Wiernik PH, Testa JR (1989) Prognostic impact of cytogenetic abnormalities in patients with de novo acute nonlymphocytic leukemia. Blood 73:263–270
13. Dastugue N, Payen C, Lafage-Pochitaloff M, Bernard P, Leroux D et al (1995) Prognostic signifcance of karyotype in de novo adult acute myeloid leukemia. Leukemia 9:1491–1498
14. Grimwade D, Walker H, Oliver F, Wheatley K, Harrison C et al (1998) The importance of diagnostic cytogenetics on outcome in AML: analysis of 1,612 patients entered into the MRC AML 10 trial. Blood 92:2322–2333
15. Slovak ML, Kopecky KJ, Cassileth PA, Harrington DH, Theil KS et al (2000) Karyotypic analysis predicts outcome of preremission and postremission therapy in adult acute myeloid leukemia: a Southwest Oncology Group/Eastern Cooperative Oncology Group study. Blood 96:4075–4083
16. Byrd JC, Mrózek K, Dodge RK, Carroll AJ, Edwards CG et al (2002) Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with de novo acute myeloid leukemia: results from Cancer and Leukemia Group B (CALGB 8461). Blood 100:4325–4336
17. Mrózek K, Heerema NA, Bloomfield CD (2004) Cytogenetics in acute leukemia. Blood Rev 18:115–136
18. Grimwade D, Walker H, Harrison G, Oliver F, Chatters S et al (2001) The predictive value of hierarchical cytogenetic classification in older adults with acute myeloid leukemia (AML): analysis of 1,065 patients entered into the United Kingdom Medical Research Council AML11 trial. Blood 98:1312–1320
19. Farag SS, Archer KJ, Mrózek K, Ruppert AS, Carroll AJ et al (2006) Pretreatment cytogenetics add to other prognostic factors predicting complete remission and long-term outcome in patients 60 years of age or older with acute myeloid leukemia: results from Cancer and Leukemia Group B 8461. Blood 108:63–73
20. Fröhling S, Schlenk RF, Kayser S, Morhardt M, Benner A et al (2006) Cytogenetics and age are major determinants of outcome in intensively treated acute myeloid leukemia patients older than 60 years: results from AMLSG trial AML HD98-B. Blood 108:3280–3288
21. van der Holt B, Breems DA, Beverloo HB, van den Berg E, Burnett AK et al (2007) Various distinctive cytogenetic abnormalities in patients with acute myeloid leukaemia aged 60 years and older express adverse prognostic value: results from a prospective clinical trial. Br J Haematol 136:96–105
22. Grimwade D, Hills RK, Moorman AV, Walker H, Chatters S et al (2010) Refinement of cytogenetic classification in acute myeloid leukemia: determination of prognostic significance of rare recurring chromosomal abnormalities among 5876 younger adult patients treated in the United Kingdom Medical Research Council trials. Blood 116:354–365
23. Gangatharan SA, Grove CS, P'ng S, O'Reilly J, Joske D et al (2013) Acute myeloid leukaemia in Western Australia 1991–2005: a retrospective population-based study of 898 patients regarding epidemiology, cytogenetics, treatment and outcome. Intern Med J 43:903–911

24. Betts DR, Ammann RA, Hirt A, Hengartner H, Beck-Popovic M et al (2007) The prognostic significance of cytogenetic aberrations in childhood acute myeloid leukaemia. A study of the Swiss Paediatric Oncology Group (SPOG). Eur J Haematol 78:468–476
25. Harrison CJ, Hills RK, Moorman AV, Grimwade DJ, Hann I et al (2010) Cytogenetics of childhood acute myeloid leukemia: United Kingdom Medical Research Council Treatment trials AML 10 and 12. J Clin Oncol 28:2674–2681
26. von Neuhoff C, Reinhardt D, Sander A, Zimmermann M, Bradtke J et al (2010) Prognostic impact of specific chromosomal aberrations in a large group of pediatric patients with acute myeloid leukemia treated uniformly according to trial AML-BFM 98. J Clin Oncol 28:2682–2689
27. Mrózek K, Carroll AJ, Maharry K, Rao KW, Patil SR et al (2008) Central review of cytogenetics is necessary for cooperative group correlative and clinical studies of adult acute leukemia: the Cancer and Leukemia Group B experience. Int J Oncol 33:239–244
28. Medeiros BC, Othus M, Estey EH, Fang M, Appelbaum FR (2014) Unsuccessful diagnostic cytogenetic analysis is a poor prognostic feature in acute myeloid leukaemia. Br J Haematol 164:245–250
29. Raimondi SC, Chang MN, Ravindranath Y, Behm FG, Gresik MV et al (1999) Chromosomal abnormalities in 478 children with acute myeloid leukemia: clinical characteristics and treatment outcome in a cooperative Pediatric Oncology Group study – POG 8821. Blood 94:3707–3716
30. Manola KN, Panitsas F, Polychronopoulou S, Daraki A, Karakosta M et al (2013) Cytogenetic abnormalities and monosomal karyotypes in children and adolescents with acute myeloid leukemia: correlations with clinical characteristics and outcome. Cancer Genet 206:63–72
31. Gray KA, Daugherty LC, Gordon SM, Seal RL, Wright MW et al (2013) Genenames.org: the HGNC resources in 2013. Nucleic Acids Res 41(Database issue):D545–D552
32. Sorensen PH, Chen CS, Smith FO, Arthur DC, Domer PH et al (1994) Molecular rearrangements of the MLL gene are present in most cases of infant acute myeloid leukemia and are strongly correlated with monocytic or myelomonocytic phenotypes. J Clin Invest 93:429–437
33. Satake N, Maseki N, Nishiyama M, Kobayashi H, Sakurai M et al (1999) Chromosome abnormalities and MLL rearrangements in acute myeloid leukemia of infants. Leukemia 13:1013–1017
34. Sam TN, Kersey JH, Linabery AM, Johnson KJ, Heerema NA et al (2012) *MLL* gene rearrangements in infant leukemia vary with age at diagnosis and selected demographic factors: a Children's Oncology Group (COG) study. Pediatr Blood Cancer 58:836–839
35. Pui C-H, Raimondi SC, Srivastava DK, Tong X, Behm FG et al (2000) Prognostic factors in infants with acute myeloid leukemia. Leukemia 14:684–687
36. Hollink IHIM, van den Heuvel-Eibrink MM, Arentsen-Peters STCJM, Pratcorona M, Abbas S et al (2011) *NUP98*/*NSD1* characterizes a novel poor prognostic group in acute myeloid leukemia with a distinct *HOX* gene expression pattern. Blood 118:3645–3656
37. Slater RM, von Drunen E, Kroes WG, Weghuis DO, van den Berg E et al (2001) t(7;12)(q36;p13) and t(7;12)(q32;p13) translocations involving *ETV6* in children 18 months of age or younger with myeloid disorders. Leukemia 15:915–920
38. Park J, Kim M, Lim J, Kim Y, Han K et al (2009) Three-way complex translocations in infant acute myeloid leukemia with t(7;12)(q36;p13): the incidence and correlation of a HLXB9 overexpression. Cancer Genet Cytogenet 191:102–105
39. Bernstein J, Dastugue N, Haas OA, Harbott J, Heerema NA et al (2000) Nineteen cases of the t(1;22)(p13;q13) acute megakaryoblastic leukaemia of infants/children and a review of 39 cases: report from a t(1;22) study group. Leukemia 14:216–218
40. Heim S, Mitelman F (2009) Nonrandom chromosome abnormalities in cancer – an overview. In: Heim S, Mitelman F (eds) Cancer cytogenetics, 3rd edn. Wiley-Blackwell, Hoboken, pp 25–43
41. Liu TX, Becker MW, Jelinek J, Wu WS, Deng M et al (2007) Chromosome 5q deletion and epigenetic suppression of the gene encoding α-catenin (*CTNNA1*) in myeloid cell transformation. Nat Med 13:78–83

42. Joslin JM, Fernald AA, Tennant TR, Davis EM, Kogan SC et al (2007) Haploinsufficiency of *EGR1*, a candidate gene in the del(5q), leads to the development of myeloid disorders. Blood 110:719–726
43. McNerney ME, Brown CD, Wang X, Bartom ET, Karmakar S et al (2013) *CUX1* is a haploinsufficient tumor suppressor gene on chromosome 7 frequently inactivated in acute myeloid leukemia. Blood 121:975–983
44. Chen C, Liu Y, Rappaport AR, Kitzing T, Schultz N et al (2014) MLL3 is a haploinsufficient 7q tumor suppressor in acute myeloid leukemia. Cancer Cell 25:652–665
45. Farag SS, Archer KJ, Mrózek K, Vardiman JW, Carroll AJ et al (2002) Isolated trisomy of chromosomes 8, 11, 13 and 21 is an adverse prognostic factor in adults with de novo acute myeloid leukemia: results from and Leukemia Group B 8461. Int J Oncol 21:1041–1051
46. Caligiuri MA, Strout MP, Schichman SA, Mrózek K, Arthur DC et al (1996) Partial tandem duplication of ALL1 as a recurrent molecular defect in acute myeloid leukemia with trisomy 11. Cancer Res 56:1418–1425
47. Dicker F, Haferlach C, Kern W, Haferlach T, Schnittger S (2007) Trisomy 13 is strongly associated with AML1/RUNX1 mutations and increased FLT3 expression in acute myeloid leukemia. Blood 110:1308–1316
48. Becker H, Maharry K, Mrózek K, Volinia S, Eisfeld A-K et al (2014) Prognostic gene mutations and distinct gene- and microRNA-expression signatures in acute myeloid leukemia with a sole trisomy 8. Leukemia 28:1754–1758
49. Johansson B, Mertens F, Mitelman F (1994) Secondary chromosomal abnormalities in acute leukemias. Leukemia 8:953–962
50. Mrózek K, Bloomfield CD (1998) Der(16)t(1;16) is a secondary chromosome aberration in at least eighteen different types of human cancer. Genes Chromosomes Cancer 23:78–80
51. Lugthart S, Gröschel S, Beverloo HB, Kayser S, Valk PJM et al (2010) Clinical, molecular, and prognostic significance of WHO type inv(3)(q21q26.2)/t(3;3)(q21;q26.2) and various other 3q abnormalities in acute myeloid leukemia. J Clin Oncol 28:3890–3898
52. Schlenk RF, Benner A, Krauter J, Büchner T, Sauerland C et al (2004) Individual patient data-based meta-analysis of patients aged 16 to 60 years with core binding factor acute myeloid leukemia: a survey of the German Acute Myeloid Leukemia Intergroup. J Clin Oncol 22:3741–3750
53. Marcucci G, Mrózek K, Ruppert AS, Maharry K, Kolitz JE et al (2005) Prognostic factors and outcome of core binding factor acute myeloid leukemia patients with t(8;21) differ from those of patients with inv(16): a Cancer and Leukemia Group B study. J Clin Oncol 23:5705–5717
54. Slack JL, Arthur DC, Lawrence D, Mrózek K, Mayer RJ et al (1997) Secondary cytogenetic changes in acute promyelocytic leukemia–prognostic importance in patients treated with chemotherapy alone and association with the intron 3 breakpoint of the PML gene: a Cancer and Leukemia Group B study. J Clin Oncol 15:1786–1795
55. Slovak ML, Gundacker H, Bloomfield CD, Dewald G, Appelbaum FR et al (2006) A retrospective study of 69 patients with t(6;9)(p23;q34) AML emphasizes the need for a prospective, multicenter initiative for rare 'poor prognosis' myeloid malignancies. Leukemia 20:1295–1297
56. Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK et al (1999) World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting-Airlie House, Virginia, November 1997. J Clin Oncol 17:3835–3849
57. Hart SM, Foroni L (2002) Core binding factor genes and human leukemia. Haematologica 87:1307–1323
58. Dayyani F, Wang J, Yeh JR, Ahn EY, Tobey E et al (2008) Loss of *TLE1* and *TLE4* from the del(9q) commonly deleted region in AML cooperates with AML1-ETO to affect myeloid cell proliferation and survival. Blood 111:4338–4347
59. Paschka P, Döhner K (2013) Core-binding factor acute myeloid leukemia: can we improve on HiDAC consolidation? Hematol Am Soc Hematol Educ Progam 2013:209–219

60. Nakamura H, Kuriyama K, Sadamori N, Mine M, Itoyama T et al (1997) Morphological subtyping of acute myeloid leukemia with maturation (AML-M2): homogeneous pink-colored cytoplasm of mature neutrophils is most characteristic of AML-M2 with t(8;21). Leukemia 11:651–655
61. Byrd JC, Dodge RK, Carroll A, Baer MR, Edwards C et al (1999) Patients with t(8;21)(q22;q22) and acute myeloid leukemia have superior failure-free and overall survival when repetitive cycles of high-dose cytarabine are administered. J Clin Oncol 17:3767–3775
62. Paschka P, Marcucci G, Ruppert AS, Mrózek K, Chen H et al (2006) Adverse prognostic significance of KIT mutations in adult acute myeloid leukemia with inv(16) and t(8;21): a Cancer and Leukemia Group B study. J Clin Oncol 24:3904–3911
63. Allen C, Hills RK, Lamb K, Evans C, Tinsley S et al (2013) The importance of relative mutant level for evaluating impact on outcome of *KIT*, *FLT3* and *CBL* mutations in core-binding factor acute myeloid leukemia. Leukemia 27:1891–1901
64. Schwind S, Edwards CG, Nicolet D, Mrózek K, Maharry K et al (2013) inv(16)/t(16;16) acute myeloid leukemia with non-type A *CBFB-MYH11* fusions associate with distinct clinical and genetic features and lack *KIT* mutations. Blood 121:385–391
65. Byrd JC, Ruppert AS, Mrózek K, Carroll AJ, Edwards CG et al (2004) Repetitive cycles of high-dose cytarabine benefit patients with acute myeloid leukemia and inv(16)(p13q22) or t(16;16)(p13;q22): results from CALGB 8461. J Clin Oncol 22:1087–1094
66. Paschka P, Du J, Schlenk RF, Gaidzik VI, Bullinger L et al (2013) Secondary genetic lesions in acute myeloid leukemia with inv(16) or t(16;16): a study of the German-Austrian AML Study Group (AMLSG). Blood 121:170–177
67. Boissel N, Leroy H, Brethon B, Philippe N, de Botton S et al (2006) Incidence and prognostic impact of c-Kit, FLT3, and Ras gene mutations in core binding factor acute myeloid leukemia (CBF-AML). Leukemia 20:965–970
68. Neubauer A, Maharry K, Mrózek K, Thiede C, Marcucci G et al (2008) Patients with acute myeloid leukemia and *RAS* mutations benefit most from postremission high-dose cytarabine: a Cancer and Leukemia Group B study. J Clin Oncol 26:4603–4609
69. Grimwade D, Biondi A, Mozziconacci MJ, Hagemeijer A, Berger R et al (2000) Characterization of acute promyelocytic leukemia cases lacking the classic t(15;17): results of the European Working Party. Blood 96:1297–1308
70. Burnett AK, Grimwade D, Solomon E, Wheatley K, Goldstone AH (1999) Presenting white blood cell count and kinetics of molecular remission predict prognosis in acute promyelocytic leukemia treated with all-trans retinoic acid: result of the randomized MRC trial. Blood 93:4131–4143
71. Grimwade D, Mrózek K (2011) Diagnostic and prognostic value of cytogenetics in acute myeloid leukemia. Hematol Oncol Clin North Am 25:1135–1161
72. Wells RA, Catzavelos C, Kamel-Reid S (1997) Fusion of retinoic acid receptor α to NuMA, the nuclear mitotic apparatus protein, by a variant translocation in acute promyelocytic leukaemia. Nat Genet 17:109–113
73. Yamamoto Y, Tsuzuki S, Tsuzuki M, Handa K, Inaguma Y et al (2010) BCOR as a novel fusion partner of retinoic acid receptor alpha in a t(X;17)(p11;q12) variant of acute promyelocytic leukemia. Blood 116:4274–4283
74. Won D, Shin SY, Park C-J, Jang S, Chi H-S et al (2013) *OBFC2A*/*RARA*: a novel fusion gene in variant acute promyelocytic leukemia. Blood 121:1432–1435
75. Chen Y, Li S, Zhou C, Li C, Ru K et al (2014) TBLR1 fuses to retinoid acid receptor α in a variant t(3;17)(q26;q21) translocation of acute promyelocytic leukemia. Blood 124:936–945
76. Catalano A, Dawson MA, Somana K, Opat S, Schwarer A et al (2007) The *PRKAR1A* gene is fused to *RARA* in a new variant acute promyelocytic leukemia. Blood 110:4073–4076
77. Grimwade D, Mistry AR, Solomon E, Guidez F (2010) Acute promyelocytic leukemia: a paradigm for differentiation therapy. Cancer Treat Res 145:219–235
78. Callens C, Chevret S, Cayuela JM, Cassinat B, Raffoux E et al (2005) Prognostic implication of FLT3 and Ras gene mutations in patients with acute promyelocytic leukemia (APL): a retrospective study from the European APL Group. Leukemia 19:1153–1160

79. Lo-Coco F, Cicconi L (2014) What is the standard regimen for patients with acute promyelocytic leukemia? Curr Hematol Malig Rep 9:138–143
80. De Botton S, Chevret S, Sanz M, Dombret H, Thomas X et al (2000) Additional chromosomal abnormalities in patients with acute promyelocytic leukaemia (APL) do not confer poor prognosis: results of APL 93 trial. Br J Haematol 111:801–806
81. Poiré X, Moser BK, Gallagher RE, Laumann K, Bloomfield CD et al (2014) Arsenic trioxide in front-line therapy of acute promyelocytic leukemia (C9710): prognostic significance of *FLT3* mutations and complex karyotype. Leuk Lymphoma 55:1523–1532
82. Dillon R, Grimwade D (2014) Prognostic significance of additional cytogenetic abnormalities and FLT3 mutations in acute promyelocytic leukemia. Leuk Lymphoma 55:1444–1446
83. Gale RE, Hills R, Pizzey AR, Kottaridis PD, Swirsky D et al (2005) Relationship between FLT3 mutation status, biologic characteristics, and response to targeted therapy in acute promyelocytic leukemia. Blood 106:3768–3776
84. Kutny MA, Moser BK, Laumann K, Feusner JH, Gamis A et al (2012) FLT3 mutation status is a predictor of early death in pediatric acute promyelocytic leukemia: a report from the Children's Oncology Group. Pediatr Blood Cancer 59:662–667
85. Noguera NI, Breccia M, Divona M, Diverio D, Costa V et al (2002) Alterations of the FLT3 gene in acute promyelocytic leukemia: association with diagnostic characteristics and analysis of clinical outcome in patients treated with the Italian AIDA protocol. Leukemia 16:2185–2189
86. Kuchenbauer F, Schoch C, Kern W, Hiddemann W, Haferlach T et al (2005) Impact of FLT3 mutations and promyelocytic leukaemia breakpoint on clinical characteristics and prognosis in acute promyelocytic leukaemia. Br J Haematol 130:196–202
87. Gallagher RE, Moser BK, Racevskis J, Poiré X, Bloomfield CD et al (2012) Treatment-influenced associations of PML-RARα mutations, FLT3 mutations, and additional chromosome abnormalities in relapsed acute promyelocytic leukemia. Blood 120:2098–2108
88. Meyer C, Hofmann J, Burmeister T, Gröger D, Park TS et al (2013) The *MLL* recombinome of acute leukemias in 2013. Leukemia 27:2165–2176
89. Krivtsov AV, Armstrong SA (2007) *MLL* translocations, histone modifications and leukaemia stem-cell development. Nat Rev Cancer 7:823–833
90. Mrózek K, Heinonen K, Lawrence D, Carroll AJ, Koduru PRK et al (1997) Adult patients with de novo acute myeloid leukemia and t(9;11)(p22;q23) have a superior outcome to patients with other translocations involving band 11q23: a Cancer and Leukemia Group B study. Blood 90:4532–4538
91. Krauter J, Wagner K, Schäfer I, Marschalek R, Meyer C et al (2009) Prognostic factors in adult patients up to 60 years old with acute myeloid leukemia and translocations of chromosome band 11q23: individual patient data-based meta-analysis of the German Acute Myeloid Leukemia Intergroup. J Clin Oncol 27:3000–3006
92. Rubnitz JE, Raimondi SC, Tong X, Srivastava DK, Razzouk BI et al (2002) Favorable impact of the t(9;11) in childhood acute myeloid leukemia. J Clin Oncol 20:2302–2309
93. Schoch C, Schnittger S, Klaus M, Kern W, Hiddemann W et al (2003) AML with 11q23/*MLL* abnormalities as defined by the WHO classification: incidence, partner chromosomes, FAB subtype, age distribution, and prognostic impact in an unselected series of 1897 cytogenetically analyzed AML cases. Blood 102:2395–2402
94. Coenen EA, Raimondi SC, Harbott J, Zimmermann M, Alonzo TA et al (2011) Prognostic significance of additional cytogenetic aberrations in 733 de novo pediatric 11q23/*MLL*-*rearranged* AML patients: results of an international study. Blood 117:7102–7111
95. Tarlock K, Alonzo TA, Moraleda PP, Gerbing RB, Raimondi SC et al (2014) Acute myeloid leukaemia (AML) with t(6;9)(p23;q34) is associated with poor outcome in childhood AML regardless of *FLT3*-ITD status: a report from the Children's Oncology Group. Br J Haematol 166:254–259
96. Ishiyama K, Takami A, Kanda Y, Nakao S, Hidaka M et al (2012) Prognostic factors for acute myeloid leukemia patients with t(6;9)(p23;q34) who underwent an allogeneic hematopoietic stem cell transplant. Leukemia 26:1416–1419

97. Sandén C, Ageberg M, Petersson J, Lennartsson A, Gullberg U (2013) Forced expression of the DEK-NUP214 fusion protein promotes proliferation dependent on upregulation of mTOR. BMC Cancer 13:440
98. Secker-Walker LM, Mehta A, Bain B (1995) Abnormalities of 3q21 and 3q26 in myeloid malignancy: a United Kingdom Cancer Cytogenetic Group study. Br J Haematol 91:490–501
99. Sun J, Konoplev SN, Wang X, Cui W, Chen SS et al (2011) De novo acute myeloid leukemia with inv(3)(q21q26.2) or t(3;3)(q21;q26.2): a clinicopathologic and cytogenetic study of an entity recently added to the WHO classification. Mod Pathol 24:384–389
100. Duchayne E, Fenneteau O, Pages MP, Sainty D, Arnoulet C et al (2003) Acute megakaryoblastic leukaemia: a national clinical and biological study of 53 adult and childhood cases by the Groupe Français d'Hématologie Cellulaire (GFHC). Leuk Lymphoma 44:49–58
101. Mrózek K, Holland KB, Pettenati MJ, Maharry K, Marcucci G et al (2009) Prognostic significance of unbalanced chromosome abnormalities used by 2008 World Health Organization (WHO) classification to define "acute myeloid leukemia (AML) with myelodysplasia-related changes" in adults: a Cancer and Leukemia Group B (CALGB) study. Blood 114:1021
102. Mrózek K, Heinonen K, Theil KS, Bloomfield CD (2002) Spectral karyotyping in patients with acute myeloid leukemia and a complex karyotype shows hidden aberrations, including recurrent overrepresentation of 21q, 11q, and 22q. Genes Chromosomes Cancer 34:137–153
103. Galván AB, Mallo M, Arenillas L, Salido M, Espinet B et al (2010) Does monosomy 5 really exist in myelodysplastic syndromes and acute myeloid leukemia? Leuk Res 34:1242–1245
104. Bloomfield CD, Goldman A, Hossfeld D, de la Chapelle A (1984) Fourth International Workshop on Chromosomes in Leukemia, 1982: clinical significance of chromosomal abnormalities in acute nonlymphoblastic leukemia. Cancer Genet Cytogenet 11:332–350
105. Swansbury GJ, Lawler SD, Alimena G, Arthur D, Berger R et al (1994) Long-term survival in acute myelogenous leukemia: a second follow-up of the Fourth International Workshop on Chromosomes in Leukemia. Cancer Genet Cytogenet 73:1–7
106. Bloomfield CD, Shuma C, Regal L, Philip PP, Hossfeld DK et al (1997) Long-term survival of patients with acute myeloid leukemia. A third follow-up of the Fourth International Workshop on Chromosomes in Leukemia. Cancer 80(suppl):2191–2198
107. Keating MJ, Smith TL, Kantarjian H, Cork A, Walters R et al (1988) Cytogenetic pattern in acute myelogenous leukemia: a major reproducible determinant of outcome. Leukemia 2:403–412
108. de Nully Brown P, Jurlander J, Pedersen-Bjergaard J, Victor MA, Geisler CH (1997) The prognostic significance of chromosomal analysis and immunophenotyping in 117 patients with de novo acute myeloid leukemia. Leuk Res 21:985–995
109. Visani G, Bernasconi P, Boni M, Castoldi GL, Ciolli S et al (2001) The prognostic value of cytogenetics is reinforced by the kind of induction/consolidation therapy in influencing the outcome of acute myeloid leukemia – analysis of 848 patients. Leukemia 15:903–909
110. O'Donnell MR, Tallman MS, Abboud CN, Altman JK, Appelbaum FR et al (2013) Acute myeloid leukemia, version 2.2013. J Natl Compr Canc Netw 11:1047–1055
111. Döhner H, Estey EH, Amadori S, Appelbaum FR, Büchner T et al (2010) Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. Blood 115:453–474
112. Larson RA, Williams SF, Le Beau MM, Bitter MA, Vardiman JW et al (1986) Acute myelomonocytic leukemia with abnormal eosinophils and inv(16) or t(16;16) has a favorable prognosis. Blood 68:1242–1249
113. Marlton P, Keating M, Kantarjian H, Pierce S, O'Brien S et al (1995) Cytogenetic and clinical correlates in AML patients with abnormalities of chromosome 16. Leukemia 9:965–971
114. Mrózek K, Prior TW, Edwards C, Marcucci G, Carroll AJ et al (2001) Comparison of cytogenetic and molecular genetic detection of t(8;21) and inv(16) in a prospective series of adults with de novo acute myeloid leukemia: a Cancer and Leukemia Group B study. J Clin Oncol 19:2482–2492

115. Schoch C, Haferlach T, Haase D, Fonatsch C, Löffler H et al (2001) Patients with de novo acute myeloid leukaemia and complex karyotype aberrations show a poor prognosis despite intensive treatment: a study of 90 patients. Br J Haematol 112:118–126
116. Mrózek K (2008) Cytogenetic, molecular genetic, and clinical characteristics of acute myeloid leukemia with a complex karyotype. Semin Oncol 35:365–377
117. Chilton L, Hills RK, Harrison CJ, Burnett AK, Grimwade D et al (2014) Hyperdiploidy with 49–65 chromosomes represents a heterogeneous cytogenetic subgroup of acute myeloid leukemia with differential outcome. Leukemia 28:321–328
118. Breems DA, Van Putten WLJ, De Greef GE, Van Zelderen-Bhola SL, Gerssen-Schoorl KBJ et al (2008) Monosomal karyotype in acute myeloid leukemia: a better indicator of poor prognosis than a complex karyotype. J Clin Oncol 26:4791–4797
119. Perrot A, Luquet I, Pigneux A, Mugneret F, Delaunay J et al (2011) Dismal prognostic value of monosomal karyotype in elderly patients with acute myeloid leukemia: a GOELAMS study of 186 patients with unfavorable cytogenetic abnormalities. Blood 118:679–685
120. Kayser S, Zucknick M, Döhner K, Krauter J, Köhne CH et al (2012) Monosomal karyotype in adult acute myeloid leukemia: prognostic impact and outcome after different treatment strategies. Blood 119:551–558
121. Cornelissen JJ, Breems D, van Putten WLJ, Gratwohl AA, Passweg JR et al (2012) Comparative analysis of the value of allogeneic hematopoietic stem-cell transplantation in acute myeloid leukemia with monosomal karyotype versus other cytogenetic risk categories. J Clin Oncol 30:2140–2146
122. Guo RJ, Atenafu EG, Craddock K, Chang H (2014) Allogeneic hematopoietic cell transplantation may alleviate the negative prognostic impact of monosomal and complex karyotypes on patients with acute myeloid leukemia. Biol Blood Marrow Transplant 20:690–695
123. Rücker FG, Schlenk RF, Bullinger L, Kayser S, Teleanu V et al (2012) *TP53* alterations in acute myeloid leukemia with complex karyotype correlate with specific copy number alterations, monosomal karyotype, and dismal outcome. Blood 119:2114–2121
124. Laï J-L, Preudhomme C, Zandecki M, Flactif M, Vanrumbeke M et al (1995) Myelodysplastic syndromes and acute myeloid leukemia with 17p deletion. An entity characterized by specific dysgranulopoiesis and a high incidence of P53 mutations. Leukemia 9:370–381
125. Rausch T, Jones DT, Zapatka M, Stütz AM, Zichner T et al (2012) Genome sequencing of pediatric medulloblastoma links catastrophic DNA rearrangements with *TP53* mutations. Cell 148:59–71
126. Heerema NA, Sanger W, Hirsch B, Magenis ME, McGavran L et al (2004) Importance of review for cooperative group cytogenetic studies, a report from the Children's Oncology Group (COG). Cytogenet Genome Res 106:136
127. Krauter J, Peter W, Pascheberg U, Heinze B, Bergmann L et al (1998) Detection of karyotypic aberrations in acute myeloblastic leukaemia: a prospective comparison between PCR/FISH and standard cytogenetics in 140 patients with de novo AML. Br J Haematol 103:72–78
128. Gruber TA, Larson Gedman A, Zhang J, Koss CS, Marada S et al (2012) An inv(16)(p13.3q24.3)-encoded *CBFA2T3-GLIS2* fusion protein defines an aggressive subtype of pediatric acute megakaryoblastic leukemia. Cancer Cell 22:683–697
129. Masetti R, Pigazzi M, Togni M, Astolfi A, Indio V et al (2013) *CBFA2T3-GLIS2* fusion transcript is a novel common feature in pediatric, cytogenetically normal AML, not restricted to FAB M7 subtype. Blood 121:3469–3472
130. Mrózek K, Marcucci G, Paschka P, Whitman SP, Bloomfield CD (2007) Clinical relevance of mutations and gene-expression changes in adult acute myeloid leukemia with normal cytogenetics: are we ready for a prognostically prioritized molecular classification? Blood 109:431–448
131. Döhner K, Schlenk RF, Habdank M, Scholl C, Rücker FG et al (2005) Mutant nucleophosmin (*NPM1*) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics: interaction with other gene mutations. Blood 106:3740–3746

132. Schnittger S, Schoch C, Kern W, Mecucci C, Tschulik C et al (2005) Nucleophosmin gene mutations are predictors of favorable prognosis in acute myelogenous leukemia with a normal karyotype. Blood 106:3733–3739
133. Thiede C, Koch S, Creutzig E, Steudel C, Illmer T et al (2006) Prevalence and prognostic impact of NPM1 mutations in 1485 adult patients with acute myeloid leukemia (AML). Blood 107:4011–4020
134. Schlenk RF, Döhner K, Krauter J, Fröhling S, Corbacioglu A et al (2008) Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. N Engl J Med 358:1909–1918
135. Becker H, Marcucci G, Maharry K, Radmacher MD, Mrózek K et al (2010) Favorable prognostic impact of *NPM1* mutations in older patients with cytogenetically normal de novo acute myeloid leukemia and associated gene- and microRNA-expression signatures: a Cancer and Leukemia Group B study. J Clin Oncol 28:596–604
136. Dufour A, Schneider F, Metzeler KH, Hoster E, Schneider S et al (2010) Acute myeloid leukemia with biallelic *CEBPA* gene mutations and normal karyotype represents a distinct genetic entity associated with a favorable clinical outcome. J Clin Oncol 28:570–577
137. Taskesen E, Bullinger L, Corbacioglu A, Sanders MA, Erpelinck CA et al (2011) Prognostic impact, concurrent genetic mutations, and gene expression features of AML with CEBPA mutations in a cohort of 1182 cytogenetically normal AML patients: further evidence for CEBPA double mutant AML as a distinctive disease entity. Blood 117:2469–2475
138. Schwind S, Maharry K, Radmacher MD, Mrózek K, Holland KB et al (2010) Prognostic significance of expression of a single microRNA, miR-181a, in cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B study. J Clin Oncol 28:5257–5264
139. Thiede C, Steudel C, Mohr B, Schaich M, Schäkel U et al (2002) Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. Blood 99:4326–4335
140. Schnittger S, Schoch C, Dugas M, Kern W, Staib P et al (2002) Analysis of FLT3 length mutations in 1003 patients with acute myeloid leukemia: correlation to cytogenetics, FAB subtype, and prognosis in the AMLCG study and usefulness as a marker for the detection of minimal residual disease. Blood 100:59–66
141. Whitman SP, Archer KJ, Feng L, Baldus C, Becknell B et al (2001) Absence of the wild-type allele predicts poor prognosis in adult de novo acute myeloid leukemia with normal cytogenetics and the internal tandem duplication of *FLT3*: a Cancer and Leukemia Group B study. Cancer Res 61:7233–7239
142. Gale RE, Green C, Allen C, Mead AJ, Burnett AK et al (2008) The impact of *FLT3* internal tandem duplication mutant level, number, size, and interaction with *NPM1* mutations in a large cohort of young adult patients with acute myeloid leukemia. Blood 111:2776–2784
143. Caligiuri MA, Strout MP, Lawrence D, Arthur DC, Baer MR et al (1998) Rearrangement of *ALL1* (*MLL*) in acute myeloid leukemia with normal cytogenetics. Cancer Res 58:55–59
144. Schnittger S, Kinkelin U, Schoch C, Heinecke A, Haase D et al (2000) Screening for MLL tandem duplication in 387 unselected patients with AML identify a prognostically unfavorable subset of AML. Leukemia 14:796–804
145. Döhner K, Tobis K, Ulrich R, Fröhling S, Benner A et al (2002) Prognostic significance of partial tandem duplications of the *MLL* gene in adult patients 16 to 60 years old with acute myeloid leukemia and normal cytogenetics: a study of the Acute Myeloid Leukemia Study Group Ulm. J Clin Oncol 20:3254–3261
146. Thol F, Damm F, Lüdeking A, Winschel C, Wagner K et al (2011) Incidence and prognostic influence of *DNMT3A* mutations in acute myeloid leukemia. J Clin Oncol 29:2889–2896
147. Marcucci G, Metzeler KH, Schwind S, Becker H, Maharry K et al (2012) Age-related prognostic impact of different types of *DNMT3A* mutations in adults with primary cytogenetically normal acute myeloid leukemia. J Clin Oncol 30:742–750
148. Renneville A, Boissel N, Nibourel O, Berthon C, Helevaut N et al (2012) Prognostic significance of DNA methyltransferase 3A mutations in cytogenetically normal acute myeloid leukemia: a study by the Acute Leukemia French Association. Leukemia 26:1247–1254

149. Marcucci G, Maharry K, Wu Y-Z, Radmacher MD, Mrózek K et al (2010) *IDH1* and *IDH2* gene mutations identify novel molecular subsets within de novo cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B study. J Clin Oncol 28:2348–2355
150. Boissel N, Nibourel O, Renneville A, Gardin C, Reman O et al (2010) Prognostic impact of isocitrate dehydrogenase enzyme isoforms 1 and 2 mutations in acute myeloid leukemia: a study by the Acute Leukemia French Association group. J Clin Oncol 28:3717–3723
151. Metzeler KH, Maharry K, Radmacher MD, Mrózek K, Margeson D et al (2011) *TET2* mutations improve the new European LeukemiaNet risk classification of acute myeloid leukemia: a Cancer and Leukemia Group B study. J Clin Oncol 29:1373–1381
152. Weissmann S, Alpermann T, Grossmann V, Kowarsch A, Nadarajah N et al (2012) Landscape of *TET2* mutations in acute myeloid leukemia. Leukemia 26:934–942
153. Tian X, Xu Y, Yin J, Tian H, Chen S et al (2014) TET2 gene mutation is unfavorable prognostic factor in cytogenetically normal acute myeloid leukemia patients with $NPM1^{+}$ and $FLT3\text{-}ITD^{-}$ mutations. Int J Hematol 100:96–104
154. Metzeler KH, Becker H, Maharry K, Radmacher MD, Kohlschmidt J et al (2011) *ASXL1* mutations identify a high-risk subgroup of older patients with primary cytogenetically normal AML within the ELN favorable genetic category. Blood 118:6920–6929
155. Schnittger S, Dicker F, Kern W, Wendland N, Sundermann J et al (2011) *RUNX1* mutations are frequent in de novo AML with noncomplex karyotype and confer an unfavorable prognosis. Blood 117:2348–2357
156. Mendler JH, Maharry K, Radmacher MD, Mrózek K, Becker H et al (2012) *RUNX1* mutations associate with poor outcome in younger and older patients with cytogenetically normal acute myeloid leukemia and with distinct gene- and microRNA-expression signatures. J Clin Oncol 30:3109–3118
157. Greif PA, Konstandin NP, Metzeler KH, Herold T, Pasalic Z et al (2012) *RUNX1* mutations in cytogenetically normal acute myeloid leukemia are associated with a poor prognosis and up-regulation of lymphoid genes. Haematologica 97:1909–1915
158. Paschka P, Marcucci G, Ruppert AS, Whitman SP, Mrózek K et al (2008) Wilms' tumor 1 gene mutations independently predict poor outcome in adults with cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B study. J Clin Oncol 26:4595–4602
159. Virappane P, Gale R, Hills R, Kakkas I, Summers K et al (2008) Mutation of the Wilms' tumor 1 gene is a poor prognostic factor associated with chemotherapy resistance in normal karyotype acute myeloid leukemia: the United Kingdom Medical Research Council Adult Leukaemia Working Party. J Clin Oncol 26:5429–5435
160. Renneville A, Boissel N, Zurawski V, Llopis L, Biggio V et al (2009) Wilms tumor 1 gene mutations are associated with a higher risk of recurrence in young adults with acute myeloid leukemia: a study from the Acute Leukemia French Association. Cancer 115:3719–3727
161. Grossmann V, Tiacci E, Holmes AB, Kohlmann A, Martelli MP et al (2011) Whole-exome sequencing identifies somatic mutations of *BCOR* in acute myeloid leukemia with normal karyotype. Blood 118:6153–6163
162. Whitman SP, Kohlschmidt J, Maharry K, Volinia S, Mrózek K et al (2014) *GAS6* expression identifies high-risk adult AML patients: potential implications for therapy. Leukemia 28:1252–1258
163. Baldus CD, Tanner SM, Ruppert AS, Whitman SP, Archer KJ et al (2003) BAALC expression predicts clinical outcome of de novo acute myeloid leukemia patients with normal cytogenetics: a Cancer and Leukemia Group B study. Blood 102:1613–1618
164. Baldus CD, Thiede C, Soucek S, Bloomfield CD, Thiel E et al (2006) *BAALC* expression and *FLT3* internal tandem duplication mutations in acute myeloid leukemia patients with normal cytogenetics: prognostic implications. J Clin Oncol 24:790–797
165. Langer C, Radmacher MD, Ruppert AS, Whitman SP, Paschka P et al (2008) High *BAALC* expression associates with other molecular prognostic markers, poor outcome, and a distinct gene-expression signature in cytogenetically normal patients younger than 60 years with acute myeloid leukemia: a Cancer and Leukemia Group B (CALGB) study. Blood 111:5371–5379

166. Schwind S, Marcucci G, Maharry K, Radmacher MD, Mrózek K et al (2010) *BAALC* and *ERG* expression levels are associated with outcome and distinct gene and microRNA expression profiles in older patients with de novo cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B study. Blood 116:5660–5669
167. Marcucci G, Baldus CD, Ruppert AS, Radmacher MD, Mrózek K et al (2005) Overexpression of the ETS-related gene, *ERG*, predicts a worse outcome in acute myeloid leukemia with normal karyotype: a Cancer and Leukemia Group B study. J Clin Oncol 23:9234–9242
168. Marcucci G, Maharry K, Whitman SP, Vukosavljevic T, Paschka P et al (2007) High expression levels of the *ETS*-related gene, *ERG*, predict adverse outcome and improve molecular risk-based classification of cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B study. J Clin Oncol 25:3337–3343
169. Metzeler KH, Dufour A, Benthaus T, Hummel M, Sauerland MC et al (2009) *ERG* expression is an independent prognostic factor and allows refined risk stratification in cytogenetically normal acute myeloid leukemia: a comprehensive analysis of *ERG*, *MN1*, and *BAALC* transcript levels using oligonucleotide microarrays. J Clin Oncol 27:5031–5038
170. Heuser M, Beutel G, Krauter J, Döhner K, von Neuhoff N et al (2006) High meningioma 1 (*MN1*) expression as a predictor for poor outcome in acute myeloid leukemia with normal cytogenetics. Blood 108:3898–3905
171. Langer C, Marcucci G, Holland KB, Radmacher MD, Maharry K et al (2009) Prognostic importance of *MN1* transcript levels, and biologic insights from *MN1*-associated gene and microRNA expression signatures in cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B study. J Clin Oncol 27:3198–3204
172. Schwind S, Marcucci G, Kohlschmidt J, Radmacher MD, Mrózek K et al (2011) Low expression of *MN1* associates with better treatment response in older patients with de novo cytogenetically normal acute myeloid leukemia. Blood 118:4188–4198
173. Alachkar H, Santhanam R, Maharry K, Metzeler KH, Huang X et al (2014) SPARC promotes leukaemic cell growth and predicts acute myeloid leukemia outcome. J Clin Invest 124:1512–1524
174. Niederwieser C, Kohlschmidt J, Volinia S, Whitman SP, Metzeler KH et al (2015) Prognostic and biologic significance of *DNMT3B* expression in older patients with cytogenetically normal primary acute myeloid leukemia. Leukemia 29:567–575
175. Eisfeld A-K, Marcucci G, Maharry K, Schwind S, Radmacher MD et al (2012) *miR-3151* interplays with its host gene *BAALC* and independently affects outcome of patients with cytogenetically normal acute myeloid leukemia. Blood 120:249–258
176. Marcucci G, Maharry KS, Metzeler KH, Volinia S, Wu Y-Z et al (2013) Clinical role of microRNAs in cytogenetically normal acute myeloid leukemia: *miR-155* upregulation independently identifies high-risk patients. J Clin Oncol 31:2086–2093
177. Röllig C, Bornhäuser M, Thiede C, Taube F, Kramer M et al (2011) Long-term prognosis of acute myeloid leukemia according to the new genetic risk classification of the European LeukemiaNet recommendations: evaluation of the proposed reporting system. J Clin Oncol 29:2758–2765
178. Mrózek K, Marcucci G, Nicolet D, Maharry KS, Becker H et al (2012) Prognostic significance of the European LeukemiaNet standardized system for reporting cytogenetic and molecular alterations in adults with acute myeloid leukemia. J Clin Oncol 30:4515–4523
179. Welch JS, Westervelt P, Ding L, Larson DE, Klco JM et al (2011) Use of whole-genome sequencing to diagnose a cryptic fusion oncogene. JAMA 305:1577–1584
180. Ding L, Ley TJ, Larson DE, Miller CA, Koboldt DC et al (2012) Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. Nature 481:506–510
181. Welch JS, Ley TJ, Link DC, Miller CA, Larson DE et al (2012) The origin and evolution of mutations in acute myeloid leukemia. Cell 150:264–278
182. Panagopoulos I, Torkildsen S, Gorunova L, Tierens A, Tjønnfjord GE et al (2014) Comparison between karyotyping-FISH-reverse transcription PCR and RNA- sequencing-fusion gene identification programs in the detection of *KAT6A-CREBBP* in acute myeloid leukemia. PLoS One 9:e96570

183. van Zutven LJ, Onen E, Velthuizen SC, van Drunen E, von Bergh AR et al (2006) Identification of *NUP98* abnormalities in acute leukemia: *JARID1A* (12p13) as a new partner gene. Genes Chromosomes Cancer 45:437–446
184. Kourlas PJ, Strout MP, Becknell B, Veronese ML, Croce CM et al (2000) Identification of a gene at 11q23 encoding a guanine nucleotide exchange factor: evidence for its fusion with *MLL* in acute myeloid leukemia. Proc Natl Acad Sci U S A 97:2145–2150
185. Shih LY, Liang DC, Fu JF, Wu JH, Wang PN et al (2006) Characterization of fusion partner genes in 114 patients with de novo acute myeloid leukemia and MLL rearrangement. Leukemia 20:218–223
186. Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ et al (2009) The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood 114:937–951
187. Whitman SP, Ruppert AS, Marcucci G, Mrózek K, Paschka P et al (2007) Long-term disease-free survivors with cytogenetically normal acute myeloid leukemia and *MLL* partial tandem duplication: a Cancer and Leukemia Group B study. Blood 109:5164–5167
188. Whitman SP, Caligiuri MA, Maharry K, Radmacher MD, Kohlschmidt J et al (2012) The *MLL* partial tandem duplication in adults aged 60 years and older with de novo cytogenetically normal acute myeloid leukemia. Leukemia 26:1713–1717
189. Gaidzik VI, Schlenk RF, Moschny S, Becker A, Bullinger L et al (2009) Prognostic impact of WT1 mutations in cytogenetically normal acute myeloid leukemia: a study of the German-Austrian AML Study Group. Blood 113:4505–4511

Part IV
Sarcomas

Chapter 14
Fusion Oncogenes of Sarcomas

Pierre Åman

Contents

14.1 Fusion Oncogenes of Sarcomas 321
14.1.1 Cytogenetic Analysis of Sarcomas 322
14.1.2 Tumour Type specificity of Sarcoma Fusion Oncogenes 323
14.2 The Fet Group of Fusion Oncogenes 323
14.3 Alveolar Rhabdomyosarcoma (ARMS) 325
14.4 Synovial Sarcoma 326
14.5 Summary 327
References 327

Abstract Fusion oncogenes are reported in many types of sarcomas. They encode protein products acting as growth factor ligands, their receptors, signal transduction components and transcription factors. The oncogenes discussed here act as regulators of transcription. FET family and *PAX3/7-FOXO1* oncoproteins bind DNA with target gene specificity causing specific tumour types with limited morphological variation. Tumours expressing *SS18-SSX* fusion proteins show larger morphological variability, perhaps reflecting that the oncoproteins act through interactions with general chromatin regulators.

Mesenchymal stem cells are suspected target cells for transformation by fusion oncogenes in sarcomas.

Keywords Fusion oncogenes • Sarcoma • Tumour type specificity • Chromosome • Rearrangements

14.1 Fusion Oncogenes of Sarcomas

The formation of fusion genes requires at least two chromosome breaks coincident in time and space. Such events yield gene fragments that may fuse into new functional constellations. The transcriptional orientation of the two partner genes must

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be compatible, and the open reading frames maintained in the fusion transcripts. Simple translocations may thus lead to functional fusion genes only if partner genes are originally transcribed in the same centromeric/telomeric direction [1, 2]. Other gene orientations require additional chromosome breaks for inversion of partner genes and are probably less frequent. Translocations and gene fusions in lymphomas and some lymphoid leukaemia are most likely caused by errors during the rearrangements of immunoglobulin and T-cell receptor genes. This process involves cell type specific rearrangement mechanisms acting at specific target genes and sequences. No such mechanisms have been reported in sarcomas although sequences related to the lymphoid rearrangement points have been reported in, or close to the translocation breakpoints of some sarcomas. The frequency of tumours caused by fusion oncogenes is further determined by the oncogenic potency. Several observations suggest that the formation of fusion oncogenes is more frequent than the incidence of the corresponding tumour, indicating that they are necessary, but not sufficient, for malignant transformation. Escape from stress responses leading to apoptosis, or oncogene induced senescence, cell or tissue type dependence, or interactions with other genetic variables are important for the final outcome after formation of a new fusion oncogene [1, 3–5].

14.1.1 Cytogenetic Analysis of Sarcomas

Chromosome translocations and gene rearrangements were first described in leukaemias and lymphomas. These early results encouraged the cytogenetic analysis of solid tumours, including sarcomas, and the identification of recurrent translocations [6–10]. Sarcomas are tumours that show morphological similarities with mesenchymal cells of different lineages and stages of development. Mesenchymal stem or precursor cells are the most likely cells of origin for this group of tumours. Close to 100 entities of sarcomas are described based on morphological and genetic criteria [11]. Tumour cells from many sarcoma cases grow relatively well in short-term cultures, and this made early cytogenetic studies possible. Most of the common sarcoma entities, were found to be characterized by complex chromosome aberrations typical for tumours with advanced genomic instability. Entities that carry simple recurrent aberrations such as translocations were also reported, and followed up on the molecular level. These studies led to the early discovery of the chromosome translocation, t(12;16), resulting in the *FUS-DDIT3* fusion oncogene (also known as *TLS-CHOP)* in myxoid liposarcoma and the t(11;22) and *EWSR1-FLI1* fusion oncogene in Ewing sarcoma [7, 9, 12–15]. A long line of fusion oncogenes have subsequently been reported in many more forms of sarcoma, and new fusions are continuously reported as next-generation methods are employed in the analysis of more tumours. Most of the currently described sarcoma fusion oncogenes encode rearranged transcription factors, but genes encoding ligands, membrane receptors and signal transduction molecules are also represented [16–18]. This chapter will focus on a discussion of a few of the transcription factors encoding sarcoma fusion oncogenes.

14.1.2 Tumour Type specificity of Sarcoma Fusion Oncogenes

A surprisingly large proportion of the sarcoma fusion oncogenes are tumour-type specific. This specificity could be explained by several models, including cell type-specific mechanisms for chromosome rearrangements, cell/tissue type dependence for survival/oncogenic activity, and phenotype instructive activity of the fusion oncogene [1, 2]. Capacity to direct the tumour phenotype has been shown for several sarcoma fusion oncogenes [19–22]. Specific cell type requirements and interactions with differentiation programmes are also important factors [19–21, 23–28]. In contrast to the specific rearrangement mechanisms behind immunoglobulin and T-cell receptor translocations in lymphoma and leukaemia, no cell type-specific rearrangement mechanisms, or target sequences have been identified in sarcoma fusion oncogenes. Furthermore, several experimental transgenic animal or cell models with cDNA copies of sarcoma fusion oncogenes at random genomic loci recapitulate the tumour phenotypes[20, 22–24, 27, 29–31]. These observations rule out cell type-specific rearrangements as a necessary mechanism behind the observed tumour type specificity of sarcoma fusion oncogenes. Cell type associated localization of chromosome territories are, however, most likely important as a risk factor for rearrangements. Formation of fusion oncogenes is discussed elsewhere in this book.

14.2 The FET Group of Fusion Oncogenes

The FET group of fusion oncogenes are found primarily in human sarcomas and leukaemia. They encode fusion oncoproteins that are considered to be primary tumour-initiating and driving factors [4, 19–22, 32]. The resulting fusion protein products contain an N-terminal domain (NTD) from one of the FET family proteins, FUS, EWSR1 or TAF15, juxtaposed to the DNA binding domains from one of many alternative transcription factor (TF) partners (Fig. 14.1).

The FET fusion oncogenes are, with a few exceptions, tumour type-specific and therefore used as diagnostic tumour markers. The FET family of fusion oncogenes continue to grow as new variant FET oncogenes, involved in more tumour entities, are continuously discovered.

There is considerable variation with regard to the breakpoint location in some FET fusion oncogenes [33–35]. Fusion transcripts of individual tumours may thus differ with regard to number of exons included from the parental genes. The N-terminal domains of the FET partners and the DNA binding domains of the TF partners are, however, always present indicating that these parts are critical for the oncogenic effect. *FUS*, *EWSR1* and *TAF15* may also functionally replace each other as fusion partners in some tumour types (Fig. 14.1). The FET fusion oncoproteins most probably act as abnormal transcription factors with FET NTDs reported to function as transactivation domains [32, 36, 37].

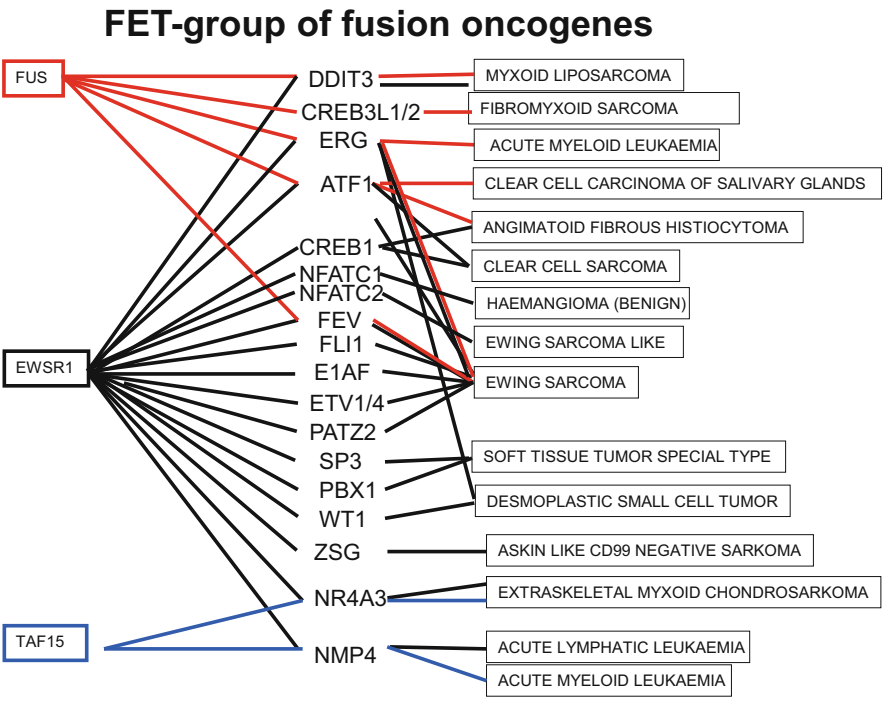

Fig. 14.1 The FET group of fusion oncogenes. The 5′ sequences of FET family genes *FUS, EWSR1* and *TAF15* are fused to genes encoding various transcription factors (*middle column*). The respective fusion genes are found in the tumour types as indicated in the right column (Note that the FET genes may replace each other in some fusion combinations. The figure is not complete as additional FET gene fusions are continuously discovered in more tumour entities)

The normal FET genes encode RNA and DNA binding proteins involved in transcriptional regulation [38–40], pre-mRNA splicing [41–43], RNA transport [44], translation [45] and DNA-repair [46, 47]. However, these functions involve the central and C-terminal protein domains that are lost in most of the fusion proteins. Analysis and structure predictions of the FET NTDs show that their structures are intrinsically disordered. Such domains are reported to function as mediators of protein-protein interactions [48]. Many proteins have also been shown to interact with the FET fusion proteins and their NTDs, including chromatin modifiers, transcription factors and cyclin dependent kinases [41, 49–55].

The full-length FET proteins were recently found as major interaction partners in pull-down experiments with FET NTDs and FUS-DDIT3. A common evolutionarily conserved "FET Binding Motif 1" (FETBM1) is present in the NTDs of all three FET proteins [56]. Specifically placed tyrosine residues, that have been reported to facilitate homotypic complex formation of FUS and similar sequences, are present in EWSR1 and TAF15 enabling formation of heterocomplexes [57]. A detailed study of the sequence requirement of the transactivating and transforming activity of EWSR1 NTD further pointed out the importance of the tyrosine residues and underscored the potential effects of their phosphorylation [58].

Experiments with deletion mutants of the full-length FUS showed that binding to the FUS NTD required amino acids 176–284, whereas residues 1–175 showed no significant affinity [56]. This observation rules out direct binding between FETBM1 sequences and indicates target structures elsewhere in the FET proteins. Combined results from several studies suggest that the FUS sequence between residues 176 and 214 forms an important target structure for FETBM1. Alignment experiments with this part of FUS, TAF15 and EWSR1 suggested that stretches containing repeats of G and RGG might form common target structures for FETBM1.

The normal recruitment of FET proteins to chromatin, genes and RNA molecules, is probably dependent on their RNA and DNA binding domains [38]. Each of the normal FET proteins binds thousands of different RNA species of several classes [59], and they also bind important protein components of transcription and RNA processing complexes [41, 51–55]. Recruitment of normal FET proteins to promoters targeted by oncogenic FET proteins may thus be an important part in deregulation of target genes.

Forced expression of FET fusion oncogenes in normal or various tumour cell lines most often results in apoptosis or cell senescence. Riggi et al. [19, 21] showed that FET oncogene- transduced mouse mesenchymal stem cells maintain proliferative capacity and are tumourigenic in mouse. Although FET oncogene-transduced human mesenchymal stem cells fail to form tumours after xenografting in mice, these data show that mesenchymal stem cells can survive and grow while expressing the fusion oncogenes. These observations suggest that FET fusion oncogenes lead to tumour formation only if they are formed in compatible cell types such as mesenchymal stem cells.

The most frequent FET oncogene-carrying tumour types are Ewing sarcoma (reviewed elsewhere in this book) and myxoid liposarcoma/round cell liposarcoma (MLS/RCLS), the latter with an incidence of 0,2 per 100,000/year [11]. The *DDIT3* partner of the myxoid liposarcoma fusion gene encodes a stress response protein induced under several stress conditions. It also has a role in the regulation of adipocyte differentiation, and forced expression of DDIT3 protein in fibrosarcoma cells has been reported to change the tumour morphology into liposarcomas [20]. *DDIT3* is also overexpressed as a result of the typical gene amplifications in well differentiated/dedifferentiated liposarcomas and may contribute to the liposarcoma phenotype of these tumours. *DDIT3* is by itself not considered an oncogene, whereas *FUS-DDIT3* transforms 3T3 cells and can turn transfected mesenchymal stem cells to liposarcoma-initiating cells in mice [19, 32].

14.3 Alveolar Rhabdomyosarcoma (ARMS)

Alveolar rhabdomyosarcoma make up around 20 % of childhood rhabdomyosarcoma cases [60]. Early cytogenetic studies, reported the recurrent t(2;13)(q36.1;q14.1) in Rhabdomyosarcomas [6, 10]. A detailed mapping of the chromosomal breakpoint regions led to the discovery of a rearranged *PAX3* gene and subsequently identified the *PAX3-FOXO1* (also known as *PAX3-FKHR*) fusion

oncogene [61–63]. Further studies showed that the less frequent t(1;13), also recurrent in ARMS, resulted in the variant *PAX7-FOXO1* fusion [63]. In the fusion proteins, the C-terminal transcriptional activation domains of PAX3 or PAX7 are replaced by the bisected forkhead transacting domain of FOXO1 retaining the N-terminal PAX3 paired box and homeodomains. The chimeric protein product of this translocation has been shown to promote cell proliferation and tumour formation by acting as an aberrant transcription factor with oncogenic properties. The fusion oncogenes have, however not been shown to cause ARMS by themselves in mesenchymal stem cells, but additional gene changes affecting *TP53* and the RAS pathway are needed [64, 65]. A number of PAX3-FOXO1 target or downstream genes have been identified. Some data suggest that the fusion oncoprotein promotes a myogenic differentiation pathway [64], and the formation of ARMS outside muscle tissue suggest that cell types other than myo-precursors may be reprogrammed by the fusion protein. Other studies show that the fusion protein blocks myocyte differentiation, partially by interfering with regulation of MyoD activity [23, 24, 27]. The normal PAX3 and PAX7 proteins have several isoforms with partially different functions and effects in myogenic differentiation, and the abnormal PAX3/7-FOXO1 proteins may cause dysregulation of this delicate system and arrest cells in a proliferative stage [66]. Normal myoblasts, with forced expression of the fusion oncoprotein, were still capable of cell fusion and myotube formation with wild type myoblasts [29]. Formation of such mixed myotubes and fibres was IL-4 receptor dependent [29]. Co-injection of myoblasts with tumour cells in mice, enhanced tumour take and growth/metastasis in an IL-4 receptor dependent manner. This suggests that cell fusions and complex interactions with normal cells are involved in the development of ARMS.

14.4 Synovial Sarcoma

Synovial sarcomas account for up to 10 % of soft-tissue sarcomas. A characteristic chromosomal translocation, t(X;18)(p11.2;q11.2), was originally reported by Turc-Carel in 1986 [8]. Molecular mapping and analysis of the breakpoint regions showed at least two different breakpoint regions on the X chromosome [67]. The translocations were subsequently shown to result in several alternative fusion genes. In the first described fusion oncogene, the 5′ end and major parts of *SS18* (also known as *SYT*) from chromosome 18 is fused to the 3′ partner *SSX1* on X [68]. Further studies showed that *SSX1* belongs to a large family of highly homologous genes and pseudogenes [69], and that several of them form fusion oncogenes with *SS18* [70]. The different *SSX* partner genes are highly similar. Still, the alternative fusion genes are associated with several morphological tumour variants, indicating functional differences in the SSX partners. This also shows that the SS18-SSX fusion oncogenes have some instructive activity that determine the tumour phenotype. As for other sarcoma fusion oncogenes, the *SS18-SSX* genes appear to be tumourigenic only in specific cell types [71, 72].

SS18–SSX encoded proteins lack DNA binding functions, but are shown to function as aberrant transcriptional regulators. *SS18* encodes a subunit of the SWI-SNF chromatin remodelling complex, and the SS18 containing fusion oncoprotein disrupts the normal formation and function of this complex [73–78]. The SSX partners interact with polycomb group protein complexes, and this activity is maintained in SS18-SSX fusion proteins [77]. Through these interactions with general chromatin remodelers and transcriptional repressors, the fusion protein may deregulate a very large numbers of genes. This very broad effect may explain the potency of this oncogene. Many direct target genes and downstream deregulated genes and functions, have been shown to be important for the oncogenic activity [73, 79, 80] and thus been proposed as therapeutic targets.

14.5 Summary

The sarcoma fusion oncogenes discussed in this chapter function as abnormal transcriptional regulators. The FET family and *PAX3/7-FOXO1* oncogenes carry DNA binding domains with sequence and target gene specificity and they cause specific tumour types with limited morphological variation. Tumours expressing *SS18-SSX* fusion proteins show larger morphological variability, perhaps reflecting the fact that these oncoproteins lack DNA binding parts but instead act by interactions with general chromatin regulators.

Stem cells of mesenchymal tissues have been proposed as the cell of origin for sarcomas. In experimental systems with forced expression of sarcoma fusion oncogenes, stem cells proliferate and form tumours, whereas other cell types fail to survive or grow. This highlights the importance of stem cells as targets for fusion oncogene-induced transformation.

References

1. Åman P (2005) Fusion oncogenes in tumor development. Semin Cancer Biol 15:236–243
2. Åman P (1999) Fusion genes in solid tumors. Semin Cancer Biol 9:303–318
3. Rodriguez R, Tornin J, Suarez C, Astudillo A, Rubio R et al (2013) Expression of FUS-CHOP fusion protein in immortalized/transformed human mesenchymal stem cells drives mixoid liposarcoma formation. Stem Cells 31(10):2061–2072
4. Rodriguez R, Rubio R, Gutierrez-Aranda I, Melen GJ, Elosua C et al (2011) FUS-CHOP fusion protein expression coupled to p53 deficiency induces liposarcoma in mouse but not in human adipose-derived mesenchymal stem/stromal cells. Stem Cells 29:179–192
5. Suva ML, Cironi L, Riggi N, Stamenkovic I (2007) Sarcomas: genetics, signalling, and cellular origins. Part 2: TET-independent fusion proteins and receptor tyrosine kinase mutations. J Pathol 213:117–130
6. Douglass EC, Valentine M, Etcubanas E, Parham D, Webber BL et al (1987) A specific chromosomal abnormality in rhabdomyosarcoma. Cytogenet Cell Genet 45:148–155

7. Turc-Carel C, Limon J, Dal Cin P, Rao U, Karakousis C et al (1986) Cytogenetic studies of adipose tissue tumors. II. Recurrent reciprocal translocation t(12;16)(q13;p11) in myxoid liposarcomas. Cancer Genet Cytogenet 23:291–299
8. Turc-Carel C, Dal Cin P, Limon J, Li F, Sandberg AA (1986) Translocation X; 18 in synovial sarcoma. Cancer Genet Cytogenet 23:93
9. Turc-Carel C, Philip I, Berger MP, Philip T, Lenoir G (1983) Chromosomal translocation (11; 22) in cell lines of Ewing's sarcoma. C R Seance Acad Sci III 296:1101–1103
10. Seidal T, Mark J, Hagmar B, Angervall L (1982) Alveolar rhabdomyosarcoma: a cytogenetic and correlated cytological and histological study. Acta Pathol Microbiol Immunol Scand A 90:345–354
11. Fletcher CDM, Unni KK, Mertens F (2000) Tumors of soft tissue and bone. IARC Press, Lyon
12. Rabbitts TH, Forster A, Larson R, Nathan P (1993) Fusion of the dominant negative transcription regulator CHOP with a novel gene FUS by translocation t(12;16) in malignant liposarcoma. Nat Genet 4:175–180
13. Crozat A, Åman P, Mandahl N, Ron D (1993) Fusion of CHOP to a novel RNA-binding protein in human myxoid liposarcoma. Nature 363:640–644
14. Delattre O, Zucman J, Plougastel B, Desmaze C, Melot T et al (1992) Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours. Nature 359:162–165
15. Åman P, Ron D, Mandahl N, Fioretos T, Heim S et al (1992) Rearrangement of the transcription factor gene CHOP in myxoid liposarcomas with t(12;16)(q13;p11). Genes Chromosomes Cancer 5:278–285
16. Wai DH, Knezevich SR, Lucas T, Jansen B, Kay RJ et al (2000) The ETV6-NTRK3 gene fusion encodes a chimeric protein tyrosine kinase that transforms NIH3T3 cells. Oncogene 19:906–915
17. Knezevich SR, McFadden DE, Tao W, Lim JF, Sorensen PH (1998) A novel ETV6-NTRK3 gene fusion in congenital fibrosarcoma. Nat Genet 18:184–187
18. Simon MP, Pedeutour F, Sirvent N, Grosgeorge J, Minoletti F et al (1997) Deregulation of the platelet-derived growth factor B-chain gene via fusion with collagen gene COL1A1 in dermatofibrosarcoma protuberans and giant-cell fibroblastoma. Nat Genet 15:95–98
19. Riggi N, Cironi L, Provero P, Suva ML, Stehle JC et al (2006) Expression of the FUS-CHOP fusion protein in primary mesenchymal progenitor cells gives rise to a model of myxoid liposarcoma. Cancer Res 66:7016–7023
20. Engstrom K, Willen H, Kabjorn-Gustafsson C, Andersson C, Olsson M et al (2006) The myxoid/round cell liposarcoma fusion oncogene FUS-DDIT3 and the normal DDIT3 induce a liposarcoma phenotype in transfected human fibrosarcoma cells. Am J Pathol 168:1642–1653
21. Riggi N, Cironi L, Provero P, Suva ML, Kaloulis K et al (2005) Development of Ewing's sarcoma from primary bone marrow-derived mesenchymal progenitor cells. Cancer Res 65:11459–11468
22. Teitell MA, Thompson AD, Sorensen PH, Shimada H, Triche TJ et al (1999) EWS/ETS fusion genes induce epithelial and neuroectodermal differentiation in NIH 3 T3 fibroblasts. Lab Invest 79:1535–1543
23. Walters ZS, Villarejo-Balcells B, Olmos D, Buist TW, Missiaglia E et al (2013) JARID2 is a direct target of the PAX3-FOXO1 fusion protein and inhibits myogenic differentiation of rhabdomyosarcoma cells. Oncogene 33:1148–1157
24. Keller C, Guttridge DC (2013) Mechanisms of impaired differentiation in rhabdomyosarcoma. FEBS J 280:4323–4334
25. Calhabeu F, Hayashi S, Morgan JE, Relaix F, Zammit PS (2013) Alveolar rhabdomyosarcoma-associated proteins PAX3/FOXO1A and PAX7/FOXO1A suppress the transcriptional activity of MyoD-target genes in muscle stem cells. Oncogene 32:651–662
26. Jothi M, Nishijo K, Keller C, Mal AK (2012) AKT and PAX3-FKHR cooperation enforces myogenic differentiation blockade in alveolar rhabdomyosarcoma cell. Cell Cycle 11:895–908

27. Charytonowicz E, Matushansky I, Domenech JD, Castillo-Martin M, Ladanyi M et al (2012) PAX7-FKHR fusion gene inhibits myogenic differentiation via NF-kappaB upregulation. Clin Transl Oncol 14:197–206
28. Riggi N, Suva ML, Stamenkovic I (2011) The cancer stem cell paradigm in Ewing's sarcoma: what can we learn about these rare cells from a rare tumor? Expert Rev Anticancer Ther 11:143–145
29. Li G, Kikuchi K, Radka M, Abraham J, Rubin BP et al (2013) IL-4 receptor blockade abrogates satellite cell – rhabdomyosarcoma fusion and prevents tumor establishment. Stem Cells 31(11):2304–2312
30. Hosoyama T, Aslam MI, Abraham J, Prajapati SI, Nishijo K et al (2011) IL-4R drives dedifferentiation, mitogenesis, and metastasis in rhabdomyosarcoma. Clin Cancer Res 17:2757–2766
31. Anderson J, Ramsay A, Gould S, Pritchard-Jones K (2001) PAX3-FKHR induces morphological change and enhances cellular proliferation and invasion in rhabdomyosarcoma. Am J Pathol 159:1089–1096
32. Zinszner H, Albalat R, Ron D (1994) A novel effector domain from the RNA-binding protein TLS or EWS is required for oncogenic transformation by CHOP. Genes Dev 8:2513–2526
33. Zoubek A, Pfleiderer C, Salzer-Kuntschik M, Amann G, Windhager R et al (1994) Variability of EWS chimaeric transcripts in Ewing tumours: a comparison of clinical and molecular data. Br J Cancer 70:908–913
34. Panagopoulos I, Mandahl N, Mitelman F, Åman P (1995) Two distinct FUS breakpoint clusters in myxoid liposarcoma and acute myeloid leukemia with the translocations t(12;16) and t(16;21). Oncogene 11:1133–1137
35. Panagopoulos I, Mandahl N, Ron D, Hoglund M, Nilbert M et al (1994) Characterization of the CHOP breakpoints and fusion transcripts in myxoid liposarcomas with the 12;16 translocation. Cancer Res 54:6500–6503
36. Sanchez-Garcia I, Rabbitts TH (1994) Transcriptional activation by TAL1 and FUS-CHOP proteins expressed in acute malignancies as a result of chromosomal abnormalities. Proc Natl Acad Sci U S A 91:7869–7873
37. Bailly RA, Bosselut R, Zucman J, Cormier F, Delattre O et al (1994) DNA-binding and transcriptional activation properties of the EWS-FLI-1 fusion protein resulting from the t(11;22) translocation in Ewing sarcoma. Mol Cell Biol 14:3230–3241
38. Blechingberg J, Luo Y, Bolund L, Damgaard CK, Nielsen AL (2012) Gene expression responses to FUS, EWS, and TAF15 reduction and stress granule sequestration analyses identifies FET-protein non-redundant functions. PLoS One 7:e46251
39. Lee J, Rhee BK, Bae GY, Han YM, Kim J (2005) Stimulation of Oct-4 activity by Ewing's sarcoma protein. Stem Cells 23:738–751
40. Thomas GR, Latchman DS (2002) The pro-oncoprotein EWS (Ewing's Sarcoma protein) interacts with the Brn-3a POU transcription factor and inhibits its ability to activate transcription. Cancer Biol Ther 1:428–432
41. Yang L, Embree LJ, Tsai S, Hickstein DD (1998) Oncoprotein TLS interacts with serine-arginine proteins involved in RNA splicing. J Biol Chem 273:27761–27764
42. Rappsilber J, Ryder U, Lamond AI, Mann M (2002) Large-scale proteomic analysis of the human spliceosome. Genome Res 12:1231–1245
43. Paronetto MP, Minana B, Valcarcel J (2011) The Ewing sarcoma protein regulates DNA damage-induced alternative splicing. Mol Cell 43:353–368
44. Zinszner H, Sok J, Immanuel D, Yin Y, Ron D (1997) TLS (FUS) binds RNA in vivo and engages in nucleo-cytoplasmic shuttling. J Cell Sci 110(Pt 15):1741–1750
45. Andersson MK, Stahlberg A, Arvidsson Y, Olofsson A, Semb H et al (2008) The multifunctional FUS, EWS and TAF15 proto-oncoproteins show cell type-specific expression patterns and involvement in cell spreading and stress response. BMC Cell Biol 9:37
46. Kuroda M, Sok J, Webb L, Baechtold H, Urano F et al (2000) Male sterility and enhanced radiation sensitivity in TLS(-/-) mice. EMBO J 19:453–462
47. Li H, Watford W, Li C, Parmelee A, Bryant MA et al (2007) Ewing sarcoma gene EWS is essential for meiosis and B lymphocyte development. J Clin Invest 117:1314–1323

48. Dyson HJ, Wright PE (2005) Intrinsically unstructured proteins and their functions. Nat Rev Mol Cell Biol 6:197–208
49. Embree LJ, Azuma M, Hickstein DD (2009) Ewing sarcoma fusion protein EWSR1/FLI1 interacts with EWSR1 leading to mitotic defects in zebrafish embryos and human cell lines. Cancer Res 69:4363–4371
50. Spahn L, Siligan C, Bachmaier R, Schmid JA, Aryee DN et al (2003) Homotypic and heterotypic interactions of EWS, FLI1 and their oncogenic fusion protein. Oncogene 22:6819–6829
51. Meissner M, Lopato S, Gotzmann J, Sauermann G, Barta A (2003) Proto-oncoprotein TLS/FUS is associated to the nuclear matrix and complexed with splicing factors PTB, SRm160, and SR proteins. Exp Cell Res 283:184–195
52. Araya N, Hirota K, Shimamoto Y, Miyagishi M, Yoshida E et al (2003) Cooperative interaction of EWS with CREB-binding protein selectively activates hepatocyte nuclear factor 4-mediated transcription. J Biol Chem 278:5427–5432
53. Rossow KL, Janknecht R (2001) The Ewing's sarcoma gene product functions as a transcriptional activator. Cancer Res 61:2690–2695
54. Bertolotti A, Bell B, Tora L (1999) The N-terminal domain of human TAFII68 displays transactivation and oncogenic properties. Oncogene 18:8000–8010
55. Bertolotti A, Lutz Y, Heard DJ, Chambon P, Tora L (1996) hTAF(II)68, a novel RNA/ssDNA-binding protein with homology to the pro-oncoproteins TLS/FUS and EWS is associated with both TFIID and RNA polymerase II. Embo J 15:5022–5031
56. Thomsen C, Grundevik P, Elias P, Stahlberg A, Åman P (2013) A conserved N-terminal motif is required for complex formation between FUS, EWSR1, TAF15 and their oncogenic fusion proteins. FASEB J 27:4965–4974
57. Kato M, Han TW, Xie S, Shi K, Du X et al (2012) Cell-free formation of RNA granules: low complexity sequence domains form dynamic fibers within hydrogels. Cell 149:753–767
58. Ng KP, Potikyan G, Savene RO, Denny CT, Uversky VN et al (2007) Multiple aromatic side chains within a disordered structure are critical for transcription and transforming activity of EWS family oncoproteins. Proc Natl Acad Sci U S A 104:479–484
59. Hoell JI, Larsson E, Runge S, Nusbaum JD, Duggimpudi S et al (2011) RNA targets of wild-type and mutant FET family proteins. Nat Struct Mol Biol 18:1428–1431
60. Pappo AS, Shapiro DN, Crist WM, Maurer HM (1995) Biology and therapy of pediatric rhabdomyosarcoma. J Clin Oncol 13:2123–2139
61. Galili N, Davis RJ, Fredericks WJ, Mukhopadhyay S, Rauscher FJ 3rd et al (1993) Fusion of a fork head domain gene to PAX3 in the solid tumour alveolar rhabdomyosarcoma. Nat Genet 5:230–235
62. Shapiro DN, Sublett JE, Li B, Downing JR, Naeve CW (1993) Fusion of PAX3 to a member of the forkhead family of transcription factors in human alveolar rhabdomyosarcoma. Cancer Res 53:5108–5112
63. Davis RJ, D'Cruz CM, Lovell MA, Biegel JA, Barr FG (1994) Fusion of PAX7 to FKHR by the variant t(1;13)(p36;q14) translocation in alveolar rhabdomyosarcoma. Cancer Res 54:2869–2872
64. Ren YX, Finckenstein FG, Abdueva DA, Shahbazian V, Chung B et al (2008) Mouse mesenchymal stem cells expressing PAX-FKHR form alveolar rhabdomyosarcomas by cooperating with secondary mutations. Cancer Res 68:6587–6597
65. Tarnowski M, Schneider G, Amann G, Clark G, Houghton P et al (2012) RasGRF1 regulates proliferation and metastatic behavior of human alveolar rhabdomyosarcomas. Int J Oncol 41:995–1004
66. Charytonowicz E, Matushansky I, Castillo-Martin M, Hricik T, Cordon-Cardo C et al (2011) Alternate PAX3 and PAX7 C-terminal isoforms in myogenic differentiation and sarcomagenesis. Clin Transl Oncol 13:194–203
67. de Leeuw B, Balemans M, Weghuis DO, Seruca R, Janz M et al (1994) Molecular cloning of the synovial sarcoma-specific translocation (X;18)(p11.2;q11.2) breakpoint. Hum Mol Genet 3:745–749

68. Clark J, Rocques PJ, Crew AJ, Gill S, Shipley J et al (1994) Identification of novel genes, SYT and SSX, involved in the t(X;18)(p11.2;q11.2) translocation found in human synovial sarcoma. Nat Genet 7:502–508
69. Gure AO, Tureci O, Sahin U, Tsang S, Scanlan MJ et al (1997) SSX: a multigene family with several members transcribed in normal testis and human cancer. Int J Cancer 72:965–971
70. Crew AJ, Clark J, Fisher C, Gill S, Grimer R et al (1995) Fusion of SYT to two genes, SSX1 and SSX2, encoding proteins with homology to the Kruppel-associated box in human synovial sarcoma. EMBO J 14:2333–2340
71. Haldar M, Hancock JD, Coffin CM, Lessnick SL, Capecchi MR (2007) A conditional mouse model of synovial sarcoma: insights into a myogenic origin. Cancer Cell 11:375–388
72. Naka N, Takenaka S, Araki N, Miwa T, Hashimoto N et al (2010) Synovial sarcoma is a stem cell malignancy. Stem Cells 28:1119–1131
73. Kadoch C, Crabtree GR (2013) Reversible disruption of mSWI/SNF (BAF) complexes by the SS18-SSX oncogenic fusion in synovial sarcoma. Cell 153:71–85
74. Garcia CB, Shaffer CM, Eid JE (2012) Genome-wide recruitment to Polycomb-modified chromatin and activity regulation of the synovial sarcoma oncogene SYT-SSX2. BMC Genomics 13:189
75. Garcia CB, Shaffer CM, Alfaro MP, Smith AL, Sun J et al (2012) Reprogramming of mesenchymal stem cells by the synovial sarcoma-associated oncogene SYT-SSX2. Oncogene 31:2323–2334
76. Barco R, Garcia CB, Eid JE (2009) The synovial sarcoma-associated SYT-SSX2 oncogene antagonizes the polycomb complex protein Bmi1. PLoS One 4:e5060
77. Soulez M, Saurin AJ, Freemont PS, Knight JC (1999) SSX and the synovial-sarcoma-specific chimaeric protein SYT-SSX co-localize with the human Polycomb group complex. Oncogene 18:2739–2746
78. Nagai M, Tanaka S, Tsuda M, Endo S, Kato H et al (2001) Analysis of transforming activity of human synovial sarcoma-associated chimeric protein SYT-SSX1 bound to chromatin remodeling factor hBRM/hSNF2 alpha. Proc Natl Acad Sci U S A 98:3843–3848
79. Su L, Cheng H, Sampaio AV, Nielsen TO, Underhill TM (2010) EGR1 reactivation by histone deacetylase inhibitors promotes synovial sarcoma cell death through the PTEN tumor suppressor. Oncogene 29:4352–4361
80. Barco R, Hunt LB, Frump AL, Garcia CB, Benesh A et al (2007) The synovial sarcoma SYT-SSX2 oncogene remodels the cytoskeleton through activation of the ephrin pathway. Mol Biol Cell 18:4003–4012

Chapter 15
Translocations in Ewing Sarcoma

Jason M. Tanner and Stephen L. Lessnick

Contents

15.1 Introduction 334
15.2 Clinical Overview 335
15.3 Translocations in Ewing Sarcoma 336
15.4 EWS/FLI 337
15.4.1 Wild-Type EWS and the FET Family of Proteins 337
15.4.2 Wild-Type FLI and the ETS Family of Transcription Factors 338
15.4.3 The EWS/FLI Fusion 339
15.4.4 Oncogenic Function of EWS/FLI 340
15.5 EWS/ERG 342
15.6 EWS/ETV1, EWS/ETV4, EWS/FEV 342
15.7 FUS/ERG and FUS/FEV 343
15.8 "Ewing-Like Sarcomas" and Their Translocations 344
15.9 Molecular Definitions of Ewing Sarcoma and Diagnostic Challenges 345
15.9.1 Defining the Disease 345
15.9.2 Challenges of Molecular Diagnosis 346
15.10 Conclusions 347
References 348

Abstract Ewing sarcoma is a bone-associated malignancy arising primarily in childhood and adolescence. It is an aggressive cancer harbouring a characteristic translocation, t(11;22)(q24.3;q12.2). This rearrangement fuses the genes *EWSR1* and *FLI1*, producing a fusion protein (EWS/FLI) that initiates an oncogenic transcription programme. Other rearrangements between similar genes have also been found to be drivers of Ewing sarcoma in a minority of cases. Understanding the

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molecular processes governed by these rearrangements promises to generate immediately actionable therapeutic strategies. This chapter discusses the defining role that translocations and their after-effects play in Ewing sarcoma.

Keywords Translocation • EWS/FLI • Ewing sarcoma • Transcription factor • Oncogenesis

15.1 Introduction

Ewing sarcoma was first described by James Ewing (Fig. 15.1) in 1921 as a tumour composed of distinctive sheets of cells with "small hyperchromatic nuclei" [1]. He noted that these tumours were distinguishable from osteogenic sarcoma by their histopathological morphology. Indeed, Ewing sarcoma continues to be characterized by its appearance as a small, round blue cell tumour, and modern molecular biology techniques have enabled scientists to elucidate many mechanistic details important for development of this tumour [2]. One particularly important discovery – made roughly 60 years after Ewing's first description of the disease – was that Ewing sarcoma harbours a recurrent set of chromosomal translocations that drive oncogenesis [3–5]. Further study of this key translocation event and its consequences have led to greater understanding of the disease and promises to provide improved therapies for those who fall victim to this malignancy. In this chapter, we discuss the biology of Ewing sarcoma with a focus on its associated translocations, including the two most common rearrangements t(11;22)(q24.3;q12.2) and t(21;22)(q22.2;q12.2) (which generate the fusion proteins EWS/FLI and EWS/ERG, respectively) as well as other, less common translocations.

Fig. 15.1 James Ewing (ca. 1890; Source: Images from the History of Medicine, National Library of Medicine; record UI: 101414702)

15.2 Clinical Overview

Ewing sarcoma is a relatively broad term for a group of tumours collectively known as the Ewing sarcoma family of tumours. (Previously referred to as "Ewing's" sarcoma, the WHO has opted to avoid possessive nomenclature; hence, "Ewing" sarcoma is the current WHO-accepted term that will be utilized in this chapter.) This family is predominantly composed of classic Ewing sarcoma, which is a bone-associated tumour that harbours one of a set of oncogenic translocations (discussed hereafter), but also includes tumours such as Askin's tumour, primitive neuroectodermal tumours (PNETs), and Ewing tumours arising in soft-tissue, known as extraosseous Ewing sarcoma [6–9]. Despite the nuances distinguishing these different members of the Ewing sarcoma family of tumours, chromosomal rearrangements are a common feature of Ewing family tumours, and are the focus of this chapter [8–11].

Ewing sarcoma is a disease of young people, occurring most commonly in children and adolescents. The mean age at diagnosis is 15 years, and ~80 % of all cases occur in patients under the age of 25 [12, 13]. For reasons that are not understood, the disease occurs at a modestly higher rate in males than females (male-to-female ratio of 1.2) (Fig. 15.2) [13, 14]. Although the disease is relatively rare, with an incidence of ~3 per million per year in the United States, Ewing sarcoma is the second most common childhood bone tumour, after osteosarcoma [15, 16]. It is most commonly encountered in patients of European ancestry, and is exceedingly uncommon in populations of African or East Asian ancestry [17–21].

Ewing sarcoma is an aggressive cancer with a high propensity for metastasis. In fact, up to 25 % of patients already have metastatic disease at the time of diagnosis [22]. This may well be an underestimation, as it is thought that many patients have undetectable micrometastatic disease at diagnosis as well. Indeed, the relapse rate is

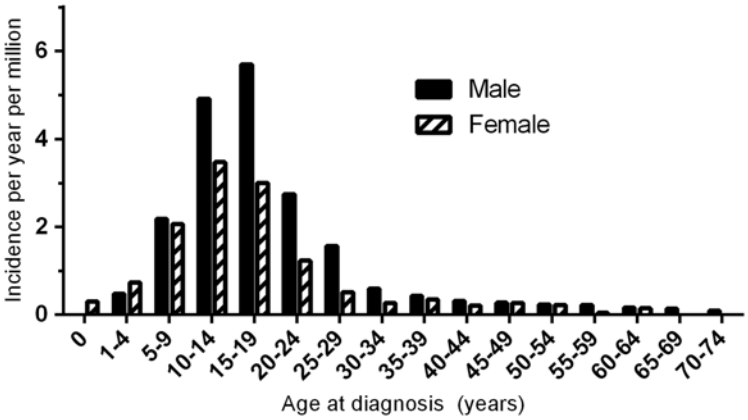

Fig. 15.2 Incidence of Ewing Sarcoma per year per million grouped by age at diagnosis (SEER data, 1973–2010) [13]

~90 % for patients who undergo surgical resection of their primary tumours without adjuvant chemotherapy [23–25]. As Ewing first observed, these tumours are often highly sensitive to radiation therapy, which was thus was a mainstay of treatment for much of the twentieth century [26, 27]. The refinement of chemotherapeutic strategies and improved surgical techniques have led to great improvements in patient survival, and current conventional treatment modalities have achieved 5-year disease-free survival rates of 60–70 % for non-metastatic disease. However, prognosis for metastatic disease remains dismal with a 5-year disease-free survival of only 10–30 % [28–30]. Moreover, survivors frequently must endure morbidities resulting from conventional anti-cancer therapy, such as severe deformities and amputations due to radical surgical resections of their tumour, and increased risk of future malignancy resulting from radiation and chemotherapy [31, 32]. Better treatments are clearly needed to provide greater survival and higher quality of life. To this end, studies continue to seek a better understanding of the molecular processes underlying Ewing sarcoma oncogenesis, including the molecular consequences of its associated chromosomal translocations.

15.3 Translocations in Ewing Sarcoma

In 1983, scientists at the Curie Institute in France identified a balanced reciprocal translocation between chromosomes 11 and 22 in patient samples and cell lines of Ewing sarcoma [3, 4, 33]. This rearrangement, t(11;22)(q24.3;q12.2), was successfully cloned several years later, and the translocation breakpoint was characterized [34]. It was revealed that this translocation resulted in an in-frame fusion of two genes: Ewing Sarcoma Breakpoint Region 1 (*EWSR1*) on chromosome 22 and Friend Leukaemia Virus Integration Site 1 (*FLI1*) on chromosome 11 [34]. The fusion protein encoded by the joining of these two genes is known as EWS/FLI. Approximately 85 % of Ewing sarcoma tumours carry this hallmark cytogenetic abnormality [9, 11, 28, 33]. The remaining 15 % of tumours carry other chromosomal rearrangements resulting in similar fusions of other genes in the same families as *EWSR1* and *FLI1* [35–39]. A list of these chromosomal rearrangements found in Ewing sarcoma is provided in Table 15.1. Details regarding each of these translocations will be discussed in the following sections.

Table 15.1 Chromosomal rearrangements found in Ewing sarcoma

Fusion	Translocation	References
EWSR1/FLI1	t (11;22)(q24;q12)	[3, 4, 33]
EWSR1/ERG	t(21;22)(q22;q12)	[38, 72]
EWSR1/ETV1	t(7;22)(p22;q12)	[35]
EWSR1/ETV4	t(7;22)(q21;q12)	[36]
EWSR1/FEV	t(2;22)(q35;q12)	[37]
FUS/ERG	t(16;21)(p11;q22)	[111]
FUS/FEV	t(2;16)(q35;p11)	[110]

15.4 EWS/FLI

15.4.1 Wild-Type EWS and the FET Family of Proteins

Prior to its cloning as part of *EWSR1/FLI1* in Ewing sarcoma, the *EWSR1* gene had not been identified and hence bears the name of the disease. *EWSR1* encodes a 656-amino acid protein called EWS. EWS is part of the FET (FUS, EWS, TAF15) family of proteins, which are involved in diverse cellular functions including gene expression and RNA processing (Fig. 15.3) [34, 40, 41].

It is ubiquitously expressed and is principally found in the nucleus, although it can be cytoplasmic or localized to the cell membrane [42–44]. The amino terminus of EWS contains a transcriptional activation region comprised of multiple pseudo-repeats rich in serine, tyrosine, glycine, and glutamine (SYGQ) (Fig. 15.3) [45–48]. This SYGQ transactivation domain is critical for interaction between EWS and RNA polymerase II; indeed, wild-type EWS has also been shown to interact with other members of the transcriptional machinery including TFIID and CREBBP/CBP/p300 [45, 46, 49]. The C-terminus of EWS contains arginine-glycine-glycine (RGG) motifs and an RNA recognition motif (RRM), possibly implicating full-length EWS in RNA binding, processing and transcription [41, 50]. The two other members of the FET family of proteins, FUS (also known as TLS) and TAF15, can also be involved in the development of other non-Ewing sarcoma cancers (Table 15.2) [51–58]. These proteins bear striking similarities to EWS, particularly with respect to the domain organization found in the N-termini of EWS and FUS [59–61].

As will be discussed in the following sections, translocations between FET genes and various partners can result in fusion proteins that alter transcriptional programmes and drive oncogenic transformation. Thus, the aforementioned interactions between FET proteins and members of the transcriptional machinery have important implications for molecular mechanisms underlying Ewing sarcoma tumourigenesis,

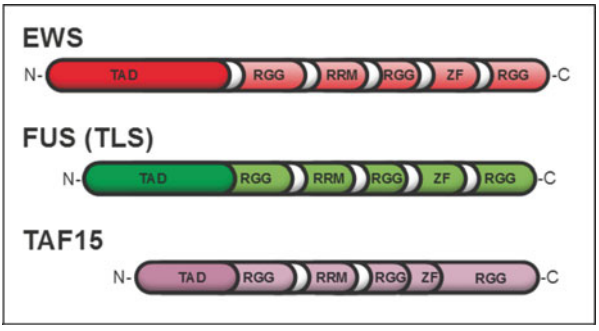

Fig. 15.3 Diagrammatic representation of FET-family proteins and their functional domains. *TAD* Transcriptional activation domain, *RGG* arginine-glycine-glycine motif, *RRM* RNA recognition motif, *ZF* zinc finger

Table 15.2 Representative non-Ewing sarcoma cancers involving translocations of FET-family proteins

	Fusion	Translocation	References
Clear cell sarcoma	EWSR1/ATF1	t(12;22)(q13;q12)	[53]
Desmoplastic small round cell tumour	EWSR1/WT1	t(11;22)(p13;q12)	[54]
Extraskeletal myxoid chondrosarcoma	EWSR1/NR4A3	t(9;22)(q22;q12)	[56]
Extraskeletal myxoid chondrosarcoma	TRF15/NR4A3	t(9;17)(q22;q11)	[52]
Myxoid liposarcoma	FUS/DDIT3	t(12;16)(q13;p11)	[51]
Myxoid liposarcoma	EWSR1/DDIT3	t(12;22)(q13;q12)	[58]
Small round cell sarcoma	EWSR1/ZNF278	t(1;22)(p36.1;q12)	[134]
Acute myelogenous leukaemia	FUS/ERG	t(16;21)(p11;q22)	[55]

as well as other cancers driven by translocations of FET genes. Recent experiments have elegantly demonstrated that FUS and EWS are able to form both homotypic and heterotypic "amyloid-like" polymers via interactions between disordered regions of polypeptides with little diversity in amino acid sequence, termed low complexity domains [49, 62]. Such aggregates could form a platform for intermolecular binding similar to molecular "velcro", leading to alteration of various cellular processes. Indeed, these polymers have been shown to bind to the C-terminal domain (CTD) of RNA polymerase II and induce transcription [49]. Accordingly, improper localization of FET proteins and their corresponding low complexity domains could disrupt gene expression at multiple loci, potentially contributing to an oncogenic phenotype. Such a model remains unproven, but is currently being actively tested.

15.4.2 Wild-Type FLI and the ETS Family of Transcription Factors

The *FLI1* gene encodes the 452 amino acid FLI protein, which is a member of the ETS (E26 transformation-specific) family of transcription factors. ETS transcription factors share a highly conserved DNA binding domain. This binding domain is known as the ETS domain, and is a winged helix-turn-helix that binds to DNA, most avidly at DNA motifs containing a core sequence of GGAA or GGAT [63, 64]. Full-length murine *Fli1* is capable of oncogenic function; indeed, the *Fli1* gene was first characterized as an integration site for the Friend murine leukaemia virus, a function from which the gene derives its name (Friend Leukaemia Virus Integration Site 1) [65]. Integration of the virus at the murine *Fli1* locus results in overexpression of *Fli1* and produces erythroleukaemia in mice [66]. Wild-type FLI appears to play important roles in haematopoiesis, particularly in megakaryocyte development [67]. Deletion of *Fli1* in mice results in dysfunctional megakaryocyte differentiation, and overexpression of *Fli1* in erythroleukaemia cells pushes them toward a megakaryocytic programme of differentiation [68, 69].

15.4.3 The EWS/FLI Fusion

To form EWS/FLI, the 5′ portion of the *EWSR1* gene and the 3′ region of the *FLI1* gene are joined together, allowing transcription of in-frame fusion transcripts and ultimately synthesis of the EWS/FLI fusion protein. The reciprocal fusion of the 5′ end of *FLI1* and the 3′ end of *EWSR1* is not expressed, and the reciprocal derivative chromosome is sometimes lost [9, 70]. Interestingly, the oncogenic *EWSR1*/*FLI1* fusion can result from several distinct translocation breakpoints occuring within introns of *EWSR1* and *FLI1* [71–73]. Classic splicing processes then generate fusion transcripts joining 5′ exons of *EWSR1* with 3′ exons of *FLI1*. EWS/FLI can thus be categorized into subtypes based upon the location of the translocation breakpoint and which exons are fused together [34]. For instance, the most commonly observed translocation in Ewing sarcoma joins exons 1–7 of *EWSR1* to exons 6–10 of *FLI1*. This rearrangement is sometimes termed a "Type I" fusion, but it is more commonly referred to simply as a "7/6" EWS/FLI fusion. Likewise, other fusions of EWS/FLI can be referred to by the exons that are fused, and a partial list of observed EWS/FLI fusions is illustrated in Fig. 15.4.

The functional significance of these subtly different EWS/FLI fusion products remains largely unknown. However, some data exist that suggest that the "7/6" EWS/FLI fusion ("Type I") is more weakly transactivating compared to other EWS/FLI fusion subtypes [74]. This distinction was thought to be potentially useful as a prognostic variable, and retrospective analyses of patient cohorts suggested that patients with "7/6" EWS/FLI fusions had better survival rates compared to patients whose tumours harboured EWS/FLI from other translocation breakpoints [75, 76]. However, recent studies have revealed that prognostic differences no longer exist within current treatment protocols [77, 78]. Hence, the functional significance of different breakpoints, if any exists at all, remains unknown.

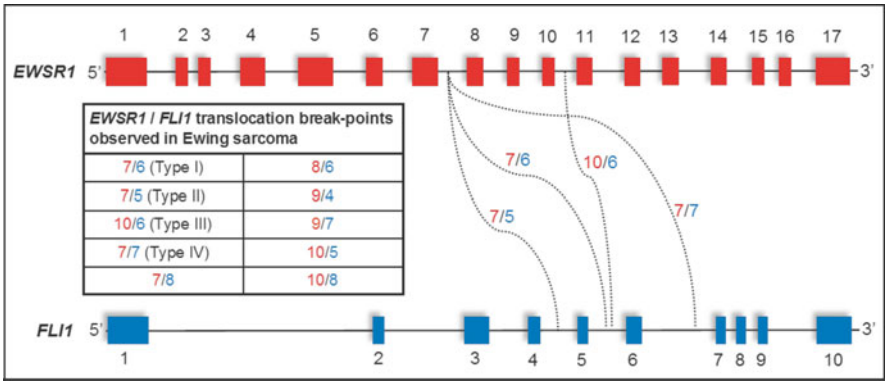

Fig. 15.4 Diagrammatic representation of *EWSR1* and *FLI1* exons. Known translocation breakpoints are indicated

15.4.4 Oncogenic Function of EWS/FLI

EWSR1 and *FLI1* genes are fused in-frame, encoding the EWS/FLI oncoprotein (Fig. 15.5). This translocation-derived oncoprotein contains the N-terminal transactivation domain of EWS fused with the DNA-binding domain of FLI, forming an oncogenic transcription factor that is indispensible for tumourigenesis [34, 42, 79–81]. The first studies implicating EWS/FLI as a driver in Ewing sarcoma observed that overexpression of EWS/FLI in NIH3T3 murine fibroblasts induced oncogenic transformation, measured by anchorage-independent growth in soft agar. This was later confirmed by experiments demonstrating the ability of EWS/FLI-expressing NIH3T3 cells to form tumours in mouse xenografts [80, 82, 83]. Furthermore, studies utilizing patient-derived Ewing sarcoma cell lines have shown that disruption of EWS/FLI expression by RNA interference (RNAi) and other means results in loss of transformation [70, 84–92]. Together, these findings clearly indicate that EWS/FLI is the driver mutation underlying Ewing sarcoma oncogenesis.

This loss of transformation is accompanied by changes in gene expression, including activation and repression of numerous EWS/FLI target genes [70, 89, 92–94]. Importantly, when EWS/FLI is reintroduced after being silenced by RNAi, the oncogenic expression profile and transformed phenotype of Ewing sarcoma are restored, indicating that EWS/FLI is at the head of an oncogenic programme of gene expression [70, 92, 94]. Studies show that thousands of genes are either

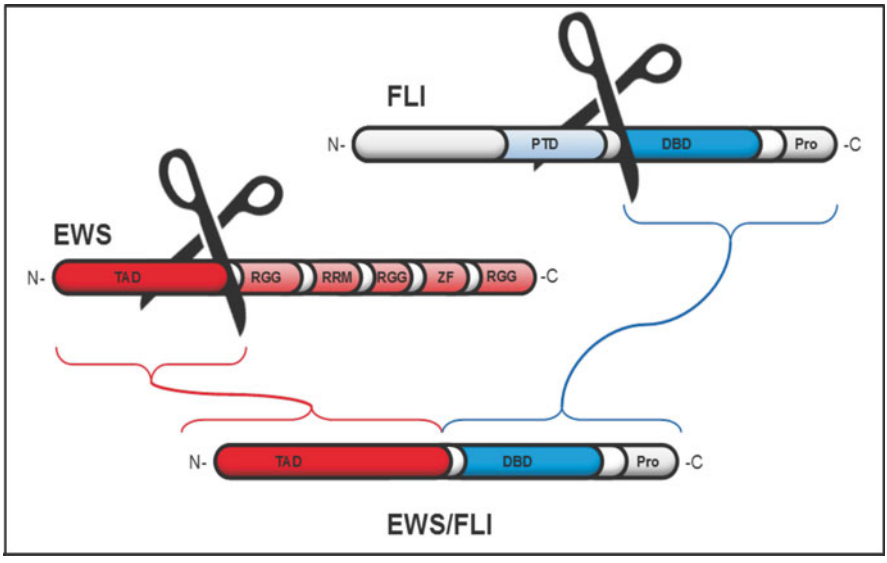

Fig. 15.5 Illustration of the EWS/FLI fusion protein, joining the N-terminal portion of EWS with the C-terminal portion of FLI. *PTD* pointed domain, *DBD* DNA binding domain, *Pro* proline-rich activation domain, *TAD* Transcriptional activation domain, *RGG* arginine-glycine-glycine motif, *RRM* RNA recognition motif, *ZF* zinc finger

upregulated or downregulated by EWS/FLI, leading to "transcriptional mayhem" [70, 81, 95]. This dysregulation of EWS/FLI target gene expression has been the focus of investigations into the mechanisms by which EWS/FLI drives tumourigenesis, and studies have revealed several EWS/FLI-regulated genes that are also required for tumourigenesis, including NR0B1, NKX2.2 and GLI1 [70, 92, 94, 96, 97].

The exact mechanisms by which EWS/FLI causes up-regulation of target genes is an area of active study. It is known that EWS/FLI alters expression of some genes in a direct manner, while it dysregulates other genes indirectly [98, 99]. Nevertheless, it has been definitively shown that the ability of EWS/FLI to bind DNA is essential for Ewing sarcoma oncogenesis [79]. Chromatin immunoprecipitation experiments followed by microarray analysis (ChIP-chip) and deep sequencing (ChIP-seq) have clearly demonstrated that EWS/FLI binds to high-affinity ETS sequences (ACCGGAAGTG) [63, 64, 100, 101]. Interestingly, it was also revealed that EWS/FLI binds to microsatellite repeats of the sequence GGAA [102, 103]. In fact, binding of EWS/FLI to microsatellites is required for upregulation of *NR0B1*, *CAV1*, and *GSTM4*; genes that are critical downstream effectors of EWS/FLI-driven tumourigenesis [102, 103].

Furthermore, as previously mentioned, it has been shown that wild-type EWS is capable of forming a molecular "velcro"-like polymer that facilitates protein-protein interactions between EWS and other proteins, including RNA polymerase II [49, 62]. The low complexity domain in the N-terminal region of wild-type EWS is retained in the EWS/FLI fusion protein, fused to the DNA-binding ETS domain of FLI. It is tempting to speculate, therefore, that the DNA-binding domain of FLI acts to re-direct the molecular "velcro" of EWS to different loci throughout the genome, leading to disruption of regulatory protein complexes and transcriptional activation of EWS/FLI target genes. For instance, GGAA microsatellite repeats could facilitate EWS/FLI polymerization as multiple DNA sequence repeats could permit EWS/FLI to bind in series, forming a scaffold of EWS low complexity domains to which coactivator complexes and transcriptional machinery (e.g., RNA polymerase II) could bind, thus upregulating that locus. Similarly, such a phenomenon could allow EWS/FLI to recruit repressive regulatory complexes to various loci, resulting in down-regulation of target genes. This model, while intriguing, remains unproven, and further testing will shed light on the true mechanisms underlying EWS/FLI-mediated transcriptional dysregulation.

EWS/FLI also down-regulates thousands of genes in Ewing sarcoma. This is particularly interesting considering the presence of the N-terminal transactivation domain of EWS in the EWS/FLI oncoprotein. The mechanisms by which such a transactivator-containing transcription factor causes direct repression of genes remains another active area of study, and several mechanistic insights have been revealed. For instance, it has been demonstrated that a corepressor complex called the Nucleosome Remodelling and Deacetylase (NuRD) complex plays an important role in repression of EWS/FLI targets. Interestingly, disruption of NuRD complex function by vorinostat treatment (a histone deacetylase inhibitor) or RNAi-mediated silencing of *CHD4* (a core NuRD component) resulted in de-repression of EWS/FLI-repressed target genes [104]. Additionally, inhibition of lysine-specific demethylase 1

(LSD1) resulted in de-repression of EWS/FLI-regulated target genes. This effect was lost upon silencing of EWS/FLI, implicating EWS/FLI-mediated disruption of associated epigenetic factors in Ewing sarcoma oncogenesis [104, 105]. Continued investigation of these phenomena is likely to generate a clearer mechanistic understanding of EWS/FLI-driven up- and down-regulation of target genes, potentially providing targets for new and better therapeutics.

15.5 EWS/ERG

In 1993 it was found that a distinct translocation event between the *EWSR1* gene and another ETS family member, *ERG* (ETS-Related Gene), also generated a fusion protein, termed EWS/ERG [38]. The t(21;22)(q22.2;q12.2) rearrangement producing this alternate fusion oncoprotein is present in approximately 10 % of Ewing tumours, making it the most common alternate translocation in Ewing sarcoma [38, 72]. Tumours carrying the EWS/ERG mutation do not carry the EWS/FLI fusion, indicating that EWS/ERG likely drives Ewing sarcoma oncogenesis in ways very similar to EWS/FLI. Indeed, the DNA-binding ETS domain of ERG is shares 98 % amino acid identity with the ETS domain of FLI, and the full-length proteins are 68 % similar [38, 106]. Furthermore, EWS/ERG-harbouring Ewing sarcoma tumours were no different compared to cases of EWS/FLI-containing tumours with respect to age at diagnosis, primary site, metastasis, as well as overall and event-free survival [107].

Like EWS/FLI, EWS/ERG induces oncogenic transformation when it is expressed in NIH3T3 cells [83]. Functionally, EWS/ERG is presumed to bind similar, if not identical, sets of loci as EWS/FLI, likely dysregulating expression of target genes in similar ways. This presumption is supported by evidence indicating that EWS/FLI and EWS/ERG dysregulate the same core subset of genes when introduced into NIH 3T3 cells, although these results must be interpreted cautiously considering the inaccuracies of this model [70, 108].

15.6 EWS/ETV1, EWS/ETV4, EWS/FEV

In addition to EWS/FLI and EWS/ERG, other EWS/ETS translocations have also been described in Ewing sarcoma. These alternate rearrangements result in the fusion of the *EWSR1* gene with *ETV1* (ETS variant gene 1), *ETV4* (ETS variant gene 4) and *FEV* (fifth Ewing sarcoma variant) (Table 15.1) [35–37, 39]. Each of these additional fusion proteins occurs in <1 % of all Ewing sarcoma cases, making them exceptionally rare. Being members of the same family of transcription factors, ERG, ETV1, ETV4 and FEV are all highly similar, particularly in their ETS DNA-binding domains. In fact, ETS domains of FLI, ERG and FEV are 98 % similar. ETV1 and ETV4 are also similar to other ETS proteins, but are more similar to each other because they have identical DNA-binding domains.

These rare alternate fusions have been less well studied than EWS/FLI. However, their structural similarities suggest that they share much of the same oncogenic functions required for Ewing sarcoma tumourigenesis. Indeed, the mutually exclusive nature of these different types of EWS/ETS fusions suggests that they may be largely interchangeable. Notwithstanding the relative paucity of data regarding these uncommon rearrangements, some functional differences have been observed in experiments utilizing NIH3T3 cells. Using this model, it was shown that EWS/FLI, EWS/ERG and EWS/FEV were capable of inducing anchorage-independent growth in soft agar assays, whereas EWS/ETV1 and EWS/ETV4 were incapable of inducing such transformation [108]. Interestingly, each fusion protein enabled tumour formation by NIH3T3 cells in murine xenografts. The mechanism and relevance of these differences remain unknown. It has also been suggested that EWS/FEV, EWS/ETV1 and EWS/ETV4 exist predominantly in extraosseous Ewing sarcoma [109]. However, insufficient data exists at the present time to draw any definitive conclusions about this potential correlation. It is also unknown whether these different fusion proteins have any significance with regard to outcome.

15.7 FUS/ERG and FUS/FEV

EWSR1 is the founding member of the FET (*FUS*, *EWSR1*, *TAF15*) family of RNA-binding proteins involved in Ewing sarcoma translocations. However, in rare instances, other members of the family are involved. Chromosomal rearrangements between *FUS* (also known as *TLS*) and *ERG* or *FEV*, both ETS family member genes, have been identified in rare cases of Ewing sarcoma [110, 111].

The FUS protein has a similar domain structure to that of EWS, containing an N-terminal transactivation domain with SYGQ repeats, and C-terminal RGG and RRM motifs (Fig. 15.5). Considering these shared structural features, it is likely that FUS/ETS fusions drive oncogenesis via mechanisms similar to those utilized by EWS/FLI. However, this hypothesis has not been thoroughly tested, in large part due to the relative scarcity of these alternate chromosomal rearrangements. Nevertheless, some functional similarities have been observed. For instance, both EWS/FLI and FUS/ERG have been shown to disrupt RNA splicing by similar mechanisms [112]. Expression of insulin-like growth factor 1 (*IGF1*) is also induced by several FET/ETS fusion proteins, including FUS/ERG [113]. However, these data must be interpreted with some caution as they are based largely on murine cells, which may lack some features important for EWS/FLI function [114].

Currently, only FUS/ERG and FUS/FEV fusions have been described, but it is possible that other FET/ETS fusions could exist in Ewing sarcoma. However, such instances would be exceedingly rare. The uncommon nature of such alternate fusions makes it difficult to elucidate whether specific rearrangements have important implications for prognosis, probability of relapse, or other factors. As mentioned before, these alternate translocations do pose a potential complication for molecular diagnosis of the disease, as a tumour that appears negative for all known

translocations may harbour an oncogenic FET/ETS rearrangement that has not yet been characterized and thus evades detection. These fusions are so scarce, however, that only a small minority of patients would be impacted by such a scenario.

15.8 "Ewing-Like Sarcomas" and Their Translocations

The existence of multiple alternate chromosomal rearrangements in Ewing sarcoma raises the question of how best to molecularly define the disease. In general, histopathological features and patient presentation give good pre-test probability for diagnosis, and definitive diagnosis commonly given by detection of CD99, a cell surface marker found on most Ewing sarcoma cells [115]. Biopsies are often subjected to molecular tests detecting the presence of the t(11;22)(q24.3;q12.2) translocation. Presence of EWS/FLI transcript are detected with RT-PCR, and translocations involving *EWSR1* are detected via breakapart FISH assays. These methods will detect almost all known FET/ETS chromosomal rearrangements in Ewing sarcoma. However, a family of tumours exists in which non-FET/ETS fusions are present (Table 15.3). These cancers are termed "Ewing-like sarcomas".

One such "Ewing-like" tumour was first reported in 2009 as a new t(20;22)(q13;q12) rearrangement between *EWSR1* and *NFATC2* (nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2) [116]. The wild-type NFATC2 protein is a member of the NFAT family of transcription factors and is a key player in T-cell and neuronal development. NFATC2 binds DNA cooperatively with Fos and Jun, members of the activator protein 1 (AP1) family of regulatory transcription factors [117–120]. Interestingly, ETS proteins and the EWS/FLI fusion protein are also capable of cooperative DNA binding with AP1 proteins [121–123]. Also, NFAT proteins, like ETS proteins, recognize DNA sequences with a core motif of GGAA/T [116]. Together these findings suggest possible shared mechanisms of oncogenesis between EWS/ETS and EWS/NFATC2 fusions.

EWSR1 can fuse to a number of other non-ETS proteins to drive formation of "Ewing-like" tumours. Another such fusion is EWS/POU5F1 [124]. POU5F1 (POU class 5 homeobox 1) is also known as OCT4 (octamer-binding transcription factor 4), and is a transcription factor important for regulating pluripotency of stem cells [125–127]. It is thought that this fusion protein functions as an aberrant transcription

Table 15.3 Non-FET/ETS chromosomal rearrangements found "Ewing-like sarcomas"

Fusion	Translocation	References
EWSR1/NFATC2	t(20;22)(q13;q12)	[116]
EWSR1/POU5F1	t(6;22)(p21;q12)	[124]
EWSR1/SMARCA5	t(4;22)(q31;q12)	[133]
EWSR1/PATZ1	t(22;22)(q12;q12)	[134]
EWSR1/SP3	t(2;22)(q31;q12)	[109]
CIC/DUX4	t(4;19)(q35;q13)	[136]
BCOR/CCNB3	inv(X)(p11.4p11.22)	[137]

factor in these tumours, transcriptionally reprogramming cells and generating an oncogenic phenotype.

Fusions between *EWSR1* and *PATZ1* (POZ (BTB) and AT Hook Containing Zinc Finger 1, also known as *ZSG*) or *SP3* are also found in some "Ewing-like" tumours [109, 128]. Both ZSG and SP3 are zinc finger-containing transcription factor proteins and, therefore, potentially function by binding DNA and allowing the EWS portion of the fusion to dysregulate gene expression profiles, similar to EWS/FLI and other Ewing sarcoma rearrangements [109, 129]. Wild-type SP3 also contains an inhibitory domain that is lost in the translocation event generating EWS/SP3, potentially contributing to its oncogenic function.

EWSR1 can also fuse with *SMARCA5* (SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, member 5), an ATPase found in various chromatin remodelling complexes [130–135]. While the EWS/SMARCA5 fusion protein does not directly bind DNA in a sequence-specific manner, it alters expression of key target genes perhaps by altering a chromatin remodelling function. Interestingly, SMARCA5 can function as part of the NuRD complex, which plays an important role in EWS/FLI-mediated repression of target genes (discussed previously) [104]. Whether any relationship exists between EWS/SMARCA5 and NuRD has not been tested.

CIC/*DUX4* and *BCOR*/*CCNB3* fusions have also been described [136, 137]. However, it has not been fully determined whether these tumours represent Ewing sarcoma, "Ewing-like" sarcoma, or a distinct type of bone sarcoma. More in-depth study of the molecular mechanisms underlying these oncogenic chromosomal rearrangements must be undertaken to answer this question. Indeed, a clear molecular-based definition of Ewing sarcoma and its variations may hinge upon achieving a clearer picture of how these fusions generate an oncogenic phenotype.

15.9 Molecular Definitions of Ewing Sarcoma and Diagnostic Challenges

15.9.1 Defining the Disease

The classic diagnostic definition of Ewing sarcoma relies largely upon histopathological features of these tumours, assessed by light microscopy and/or immunohistochemistry [138, 139]. This cancer appears as a small, round cell cancer with hyperchromatic nuclei when viewed by light microscopy after H&E staining [140]. Immunohistochemical staining often reveals high levels of CD99 at the cell membrane, and is used as another diagnostic marker of Ewing sarcoma cells [115, 138].

Additionally, the presence of a balanced translocation involving *EWSR1* and one of the *ETS* family of transcription factors are considered pathognomonic for the disease [138]. However, as discussed in this chapter, a number of different translocations involving FET family members other than *EWSR1* (e.g., *FUS*) also exist.

Additionally, several "Ewing-like" cancers have been found with fusions of EWSR1 to non-ETS proteins. These alternate molecular lesions, rare as they may be, add complexity to the question of how to properly define this disease and its variations.

Generally, Ewing sarcoma can be broadly subdivided into three groups, based on the type of translocation present in the tumour cells: (1) *EWSR1*/*FLI1* and functionally similar translocations (i.e., FET/ETS fusions), (2) non-FET/ETS fusions (e.g., EWS/SMARCA5), and (3) totally distinct translocations (e.g., *CIC*/*DUX4*). Furthermore, tumours of EWS/FLI and other FET/ETS fusions (e.g., EWS/ERG) can be considered classic Ewing sarcoma, while rarer, non-FET/ETS fusions and distinct translocations can be generally termed "Ewing-like" sarcomas. These definitions provide a useful categorical structure for the various molecular lesions driving oncogenesis in these tumours, but definitions will be continuously refined as our understanding of the molecular mechanisms of this disease expands.

Accurate and useful definitions are important insomuch as they may assist in grouping patients in clinically useful ways, such as into groups receiving different treatments or with different prognoses. These goals are especially challenging considering the rarity of non-EWS/FLI fusion variants, and little variation currently exists in the clinical management of different types of fusions.

15.9.2 Challenges of Molecular Diagnosis

The existence of alternate chromosomal rearrangements has clear implications for the diagnosis of Ewing sarcoma. Current diagnostic methods utilized to identify the EWS/FLI fusion may not identify the less common translocations. For instance, breakapart FISH (fluorescence in situ hybridization) probes for *EWSR1* are commonly utilized to determine that a translocation involving EWSR1 exists and are, thus, useful for detecting the most common rearrangements in Ewing sarcoma (i.e., EWS/FLI and EWS/ERG) [138, 139]. This method, however, is unable to detect Ewing sarcoma driven by non-*EWSR1* rearrangements, such as the rarer FUS/ERG and FUS/FEV fusions (Fig. 15.6).

Reverse-transcriptase (RT)-PCR assays have also been utilized to detect the fusion transcript [139]. Such an approach suffers from the same weakness as the EWSR1 breakapart FISH assay in that it is unable to detect transcripts of all possible gene fusions. For instance, primers designed to amplify specific *EWSR1*/*FLI1* fusions will not anneal to *EWSR1*/*ERG* or other alternate transcripts. Despite this weakness, one potential benefit to using a PCR-based assay is the ability to detect specific breakpoints, although this may not be clinically useful, as discussed earlier [75, 76, 78].

Hence, the rare cases of Ewing sarcoma driven by alternate translocations may theoretically result in delayed or incorrect diagnosis in uncommon cases. Clearly, the correct diagnosis of Ewing sarcoma must not rely on one single test but rather on a collection of various criteria, including patient presentation, imaging studies (e.g., X-ray, CT, MRI), histopathology, and pathognomonic molecular lesions such

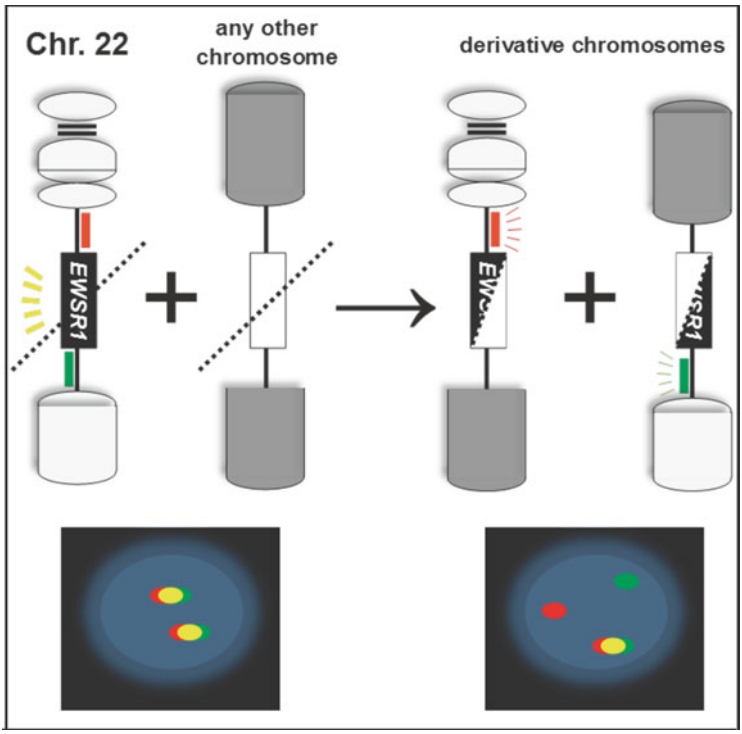

Fig. 15.6 Diagrammatic representation of a breakapart fluorescence in situ hybridization (FISH) assay for *EWSR1*. Fluorescent red and green probes flank the *EWSR1* gene. Intact *EWSR1* with both probes appears *yellow*. A translocation splits the gene, resulting in split *red* and *green* signals. In diploid cells, separate *red* and *green* signals result from the split chromosome, and the normal second allele appears *yellow*

as EWS/FLI. Such a practice of integrating distinct pieces of data to come to a definitive diagnosis is the current practice, allowing for prompt and accurate diagnosis in almost all cases.

15.10 Conclusions

Although it is rare compared to other malignancies, Ewing sarcoma is a devastating disease affecting many young people, resulting in many years of life lost to morbidity and mortality. Over the past 30 years, scientists have made great strides in understanding the molecular mechanisms underlying this cancer. Nevertheless, the increased knowledge gained through studying the cellular and molecular biology of this disease has not yet led to improvements in clinical management. Current standards of care rely on conventional therapies like surgery and chemotherapy, and

improved usage of these treatment modalities have achieved remarkable success in overall survival. No molecularly targeted therapy has been found to be efficacious against Ewing sarcoma, despite increased understanding of the molecular biology of the disease.

The EWS/FLI fusion protein, and the other fusions found in Ewing sarcoma, clearly offer a unique pathogenic feature of this disease that could be targeted. However, transcription factors have proven to be extraordinarily challenging targets for inhibition, often earning them the epithet "undruggable". Thus most efforts have focused on developing deeper understanding of the functions of critical effectors of EWS/FLI-driven oncogenesis. Although progress has been slow, a few promising targets have recently emerged [141]. Future work will continue to pursue a clearer understanding of the oncogenic consequences of the chromosomal rearrangements discussed in this chapter. Understanding why these translocations drive oncogenesis will assist in developing new therapies, likely increasing the odds of survival and bettering post-survival quality of life in these patients.

References

1. Ewing J (1921) Diffuse endothelioma of bone. Proc N Y Phytopathol Soc 21:17–24
2. Stiller CA, Bielack SS, Jundt G, Steliarova-Foucher E (2006) Bone tumours in European children and adolescents, 1978–1997. Report from the Automated Childhood Cancer Information System project. Eur J Cancer 42:2124–2135
3. Aurias A, Rimbaut C, Buffe D, Dubousset J, Mazabraud A (1983) Translocation of chromosome 22 in Ewing's sarcoma. C R Seances Acad Sci III Sci Vie 296:1105–1107
4. Turc-Carel C, Philip I, Berger MP, Philip T, Lenoir G (1983) Chromosomal translocation (11; 22) in cell lines of Ewing's sarcoma. C R Seances Acad Sci III Sci Vie 296:1101–1103
5. Janknecht R (2005) EWS-ETS oncoproteins: the linchpins of Ewing tumors. Gene 363:1–14
6. Horowitz ME, Malawer MM, Woo SY, Hicks MJ (1997) In: Pizzo PA, Poplack DG (eds) Principles and practice of pediatric oncology. Lippincott-Raven Publishers, Philadelphia, pp 831–863
7. Kimber C, Michalski A, Spitz L, Pierro A (1998) Primitive neuroectodermal tumours: anatomic location, extent of surgery, and outcome. J Pediatr Surg 33:39–41
8. Grier HE (1997) The Ewing family of tumors. Ewing's sarcoma and primitive neuroectodermal tumors. Pediatr Clin N Am 44:991–1004
9. Turc-Carel C, Philip I, Berger MP, Philip T, Lenoir GM (1984) Chromosome study of Ewing's sarcoma (ES) cell lines. Consistency of a reciprocal translocation t(11;22)(q24;q12). Cancer Genet Cytogenet 12:1–19
10. Kovar H (1998) Ewing's sarcoma and peripheral primitive neuroectodermal tumors after their genetic union. Curr Opin Oncol 10:334–342
11. Whang-Peng J et al (1984) Chromosome translocation in peripheral neuroepithelioma. N Engl J Med 311:584–585
12. Ries LAG et al (2001) SEER cancer statistics review, 1973–1998. National Cancer Institute, Bethesda
13. Surveillance, E., and End Results (SEER) Program (www.seer.cancer.gov) SEER*Stat Database: Incidence – SEER 9 Regs Research Data, Nov 2012 Sub (1973–2010) <Katrina/Rita Population Adjustment> – Linked to county attributes – total U.S., 1969–2011 Counties, National Cancer Institute, DCCPS, Surveillance Research Program, Surveillance Systems Branch, released April 2013, based on the November 2012 submission. Accessed February 2013

14. Stiller CA et al (2006) Cancer incidence and survival in European adolescents (1978–1997). Report from the Automated Childhood Cancer Information System project. Eur J Cancer 42:2006–2018
15. Denny CT (1998) Ewing's sarcoma – a clinical enigma coming into focus. J Pediatr Hematol Oncol 20:421–425
16. Paulussen M, Frohlich B, Jurgens H (2001) Ewing tumour: incidence, prognosis and treatment options. Pediatr Drugs 3:899–913
17. Parkin DM, Stiller CA, Nectoux J (1993) International variations in the incidence of childhood bone tumours. Int J Cancer 53:371–376
18. Polednak AP (1985) Primary bone cancer incidence in black and white residents of New York State. Cancer 55:2883–2888
19. Bahebeck J et al (2003) Bone tumours in Cameroon: incidence, demography and histopathology. Int Orthop 27:315–317
20. Settakorn J et al (2006) Spectrum of bone tumors in Chiang Mai University Hospital, Thailand according to WHO classification 2002: a study of 1,001 cases. J Med Assoc Thai 89:780–787
21. Guo W, Xu W, Huvos AG, Healey JH, Feng C (1999) Comparative frequency of bone sarcomas among different racial groups. Chin Med J (Engl) 112:1101–1104
22. Terrier P, Llombart-Bosch A, Contesso G (1996) Small round blue cell tumors in bone: prognostic factors correlated to Ewing's sarcoma and neuroectodermal tumors. Semin Diagn Pathol 13:250–257
23. Wang CC, Schulz MD (1953) Ewing's sarcoma; a study of fifty cases treated at the Massachusetts General Hospital, 1930–1952 inclusive. N Engl J Med 248:571–576
24. Dahlin DC, Coventry MB, Scanlon PW (1961) Ewing's sarcoma. A critical analysis of 165 cases. J Bone Joint Surg Am 43-A:185–192
25. Lahl M, Fisher VL, Laschinger K (2008) Ewing's sarcoma family of tumors: an overview from diagnosis to survivorship. Clin J Oncol Nurs 12:89–97. doi:10.1188/08.CJON.89-97
26. Lee ES (1971) Treatment of bone sarcoma. Proc R Soc Med 64:1179–1180
27. Rosen G et al (1978) Curability of Ewing's sarcoma and considerations for future therapeutic trials. Cancer 41:888–899
28. Sankar S, Lessnick SL (2011) Promiscuous partnerships in Ewing's sarcoma. Cancer Genet 204:351–365. doi:10.1016/j.cancergen.2011.07.008
29. Linabery AM, Ross JA (2008) Childhood and adolescent cancer survival in the US by race and ethnicity for the diagnostic period 1975–1999. Cancer 113:2575–2596
30. Randall RL et al (2010) Is there a predisposition gene for Ewing's sarcoma? J Oncol 2010:397632. doi:10.1155/2010/397632
31. May WA et al (2013) Characterization and drug resistance patterns of Ewing's sarcoma family tumor cell lines. PLoS One 8:e80060. doi:10.1371/journal.pone.0080060
32. Potratz J, Dirksen U, Jurgens H, Craft A (2012) Ewing sarcoma: clinical state-of-the-art. Pediatr Hematol Oncol 29:1–11. doi:10.3109/08880018.2011.622034
33. Turc-Carel C et al (1988) Chromosomes in Ewing's sarcoma. I. An evaluation of 85 cases of remarkable consistency of t(11;22)(q24;q12). Cancer Genet Cytogenet 32:229–238
34. Delattre O et al (1992) Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours. Nature 359:162–165
35. Jeon IS et al (1995) A variant Ewing's sarcoma translocation (7;22) fuses the EWS gene to the ETS gene ETV1. Oncogene 10:1229–1234
36. Kaneko Y et al (1996) Fusion of an ETS-family gene, EIAF, to EWS by t(17;22)(q12;q12) chromosome translocation in an undifferentiated sarcoma of infancy. Gene Chromosome Cancer 15:115–121
37. Peter M et al (1997) A new member of the ETS family fused to EWS in Ewing tumors. Oncogene 14:1159–1164
38. Sorensen PH et al (1994) A second Ewing's sarcoma translocation, t(21;22), fuses the EWS gene to another ETS-family transcription factor, ERG. Nat Genet 6:146–151. doi:10.1038/ng0294-146

39. Urano F, Umezawa A, Hong W, Kikuchi H, Hata J (1996) A novel chimera gene between EWS and E1A-F, encoding the adenovirus E1A enhancer-binding protein, in extraosseous Ewing's sarcoma. Biochem Biophys Res Commun 219:608–612
40. Aman P et al (1996) Expression patterns of the human sarcoma-associated genes FUS and EWS and the genomic structure of FUS. Genomics 37:1–8
41. Ohno T et al (1994) The EWS gene, involved in Ewing family of tumors, malignant melanoma of soft parts and desmoplastic small round cell tumors, codes for an RNA binding protein with novel regulatory domains. Oncogene 9:3087–3097
42. May WA et al (1993) The Ewing's sarcoma EWS/FLI-1 fusion gene encodes a more potent transcriptional activator and is a more powerful transforming gene than FLI-1. Mol Cell Biol 13:7393–7398
43. Sutherland HG et al (2001) Large-scale identification of mammalian proteins localized to nuclear sub-compartments. Hum Mol Genet 10:1995–2011
44. Zakaryan RP, Gehring H (2006) Identification and characterization of the nuclear localization/retention signal in the EWS proto-oncoprotein. J Mol Biol 363:27–38
45. Araya N et al (2003) Cooperative interaction of EWS with CREB-binding protein selectively activates hepatocyte nuclear factor 4-mediated transcription. J Biol Chem 278:5427–5432
46. Bertolotti A et al (1998) EWS, but not EWS-FLI-1, is associated with both TFIID and RNA polymerase II: interactions between two members of the TET family, EWS and hTAFII68, and subunits of TFIID and RNA polymerase II complexes. Mol Cell Biol 18:1489–1497
47. Petermann R et al (1998) Oncogenic EWS-Fli1 interacts with hsRPB7, a subunit of human RNA polymerase II. Oncogene 17:603–610
48. Rossow KL, Janknecht R (2001) The Ewing's sarcoma gene product functions as a transcriptional activator. Cancer Res 61:2690–2695
49. Kwon I et al (2013) Phosphorylation-regulated binding of RNA polymerase II to fibrous polymers of low-complexity domains. Cell 155:1049–1060. doi:10.1016/j.cell.2013.10.033
50. Deloulme JC, Prichard L, Delattre O, Storm DR (1997) The prooncoprotein EWS binds calmodulin and is phosphorylated by protein kinase C through an IQ domain. J Biol Chem 272:27369–27377
51. Crozat A, Aman P, Mandahl N, Ron D (1993) Fusion of CHOP to a novel RNA-binding protein in human myxoid liposarcoma. Nature 363:640–644
52. Panagopoulos I et al (1999) Fusion of the RBP56 and CHN genes in extraskeletal myxoid chondrosarcomas with translocation t(9;17)(q22;q11). Oncogene 18:7594–7598
53. Zucman J et al (1993) EWS and ATF-1 gene fusion induced by t(12;22) translocation in malignant melanoma of soft parts. Nat Genet 4:341–345
54. Ladanyi M, Gerald W (1994) Fusion of the EWS and WT1 genes in the desmoplastic small round cell tumor. Cancer Res 54:2837–2840
55. Martini A et al (2002) Recurrent rearrangement of the Ewing's sarcoma gene, EWSR1, or its homologue, TAF15, with the transcription factor CIZ/NMP4 in acute leukemia. Cancer Res 62:5408–5412
56. Gill S et al (1995) Fusion of the EWS gene to a DNA segment from 9q22-31 in a human myxoid chondrosarcoma. Gene Chromosome Cancer 12:307–310
57. Sjogren H et al (2000) Fusion of the NH2-terminal domain of the basic helix-loop-helix protein TCF12 to TEC in extraskeletal myxoid chondrosarcoma with translocation t(9;15)(q22;q21). Cancer Res 60:6832–6835
58. Panagopoulos I et al (1996) Fusion of the EWS and CHOP genes in myxoid liposarcoma. Oncogene 12:489–494
59. Bertolotti A, Lutz Y, Heard DJ, Chambon P, Tora L (1996) hTAF(II)68, a novel RNA/ssDNA-binding protein with homology to the pro-oncoproteins TLS/FUS and EWS is associated with both TFIID and RNA polymerase II. EMBO J 15:5022–5031
60. Morohoshi F, Arai K, Takahashi EI, Tanigami A, Ohki M (1996) Cloning and mapping of a human RBP56 gene encoding a putative RNA binding protein similar to FUS/TLS and EWS proteins. Genomics 38:51–57

61. Stolow DT, Haynes SR (1995) Cabeza, a Drosophila gene encoding a novel RNA binding protein, shares homology with EWS and TLS, two genes involved in human sarcoma formation. Nucleic Acids Res 23:835–843
62. Kato M et al (2012) Cell-free formation of RNA granules: low complexity sequence domains form dynamic fibers within hydrogels. Cell 149:753–767. doi:10.1016/j.cell.2012.04.017
63. Seth A, Watson DK (2005) ETS transcription factors and their emerging roles in human cancer. Eur J Cancer 41:2462–2478
64. Sharrocks AD (2001) The ETS-domain transcription factor family. Nat Rev Mol Cell Biol 2:827–837
65. Ben-David Y, Giddens EB, Bernstein A (1990) Identification and mapping of a common proviral integration site Fli-1 in erythroleukemia cells induced by Friend murine leukemia virus. Proc Natl Acad Sci U S A 87:1332–1336
66. Ben-David Y, Giddens EB, Letwin K, Bernstein A (1991) Erythroleukemia induction by Friend murine leukemia virus: insertional activation of a new member of the ets gene family, Fli-1, closely linked to c-ets-1. Genes Dev 5:908–918
67. Bastian LS, Kwiatkowski BA, Breininger J, Danner S, Roth G (1999) Regulation of the megakaryocytic glycoprotein IX promoter by the oncogenic Ets transcription factor Fli-1. Blood 93:2637–2644
68. Athanasiou M et al (1996) Increased expression of the ETS-related transcription factor FLI-1/ERGB correlates with and can induce the megakaryocytic phenotype. Cell Growth Differ 7:1525–1534
69. Hart A et al (2000) Fli-1 is required for murine vascular and megakaryocytic development and is hemizygously deleted in patients with thrombocytopenia. Immunity 13:167–177
70. Smith R et al (2006) Expression profiling of EWS/FLI identifies NKX2.2 as a critical target gene in Ewing's sarcoma. Cancer Cell 9:405–416. doi:10.1016/j.ccr.2006.04.004
71. Zucman J et al (1992) Cloning and characterization of the Ewing's sarcoma and peripheral neuroepithelioma t(11;22) translocation breakpoints. Gene Chromosome Cancer 5:271–277
72. Zucman J et al (1993) Combinatorial generation of variable fusion proteins in the Ewing family of tumours. EMBO J 12:4481–4487
73. Zucman-Rossi J, Legoix P, Victor JM, Lopez B, Thomas G (1998) Chromosome translocation based on illegitimate recombination in human tumors. Proc Natl Acad Sci U S A 95:11786–11791
74. Lin PP et al (1999) Differential transactivation by alternative EWS-FLI1 fusion proteins correlates with clinical heterogeneity in Ewing's sarcoma. Cancer Res 59:1428–1432
75. de Alava E et al (1998) EWS-FLI1 fusion transcript structure is an independent determinant of prognosis in Ewing's sarcoma. J Clin Oncol 16:1248–1255
76. Zoubek A et al (1996) Does expression of different EWS chimeric transcripts define clinically distinct risk groups of Ewing tumor patients? J Clin Oncol 14:1245–1251
77. Le Deley MC et al (2010) Impact of EWS-ETS fusion type on disease progression in Ewing's sarcoma/peripheral primitive neuroectodermal tumor: prospective results from the cooperative Euro-E.W.I.N.G. 99 trial. J Clin Oncol Off J Am Soc Clin Oncol 28:1982–1988. doi:10.1200/JCO.2009.23.3585
78. van Doorninck JA et al (2010) Current treatment protocols have eliminated the prognostic advantage of type 1 fusions in Ewing sarcoma: a report from the Children's Oncology Group. J Clin Oncol Off J Am Soc Clin Oncol 28:1989–1994. doi:10.1200/JCO.2009.24.5845
79. Lessnick SL, Braun BS, Denny CT, May WA (1995) Multiple domains mediate transformation by the Ewing's sarcoma EWS/FLI- 1 fusion gene. Oncogene 10:423–431
80. May WA et al (1993) Ewing sarcoma 11;22 translocation produces a chimeric transcription factor that requires the DNA-binding domain encoded by FLI1 for transformation. Proc Natl Acad Sci U S A 90:5752–5756
81. Hancock JD, Lessnick SL (2008) A transcriptional profiling meta-analysis reveals a core EWS-FLI gene expression signature. Cell Cycle 7:250–256

82. Teitell MA et al (1999) EWS/ETS fusion genes induce epithelial and neuroectodermal differentiation in NIH 3T3 fibroblasts. Lab Invest 79:1535–1543
83. Thompson AD, Teitell MA, Arvand A, Denny CT (1999) Divergent Ewing's sarcoma EWS/ETS fusions confer a common tumorigenic phenotype on NIH3T3 cells. Oncogene 18:5506–5513. doi:10.1038/sj.onc.1202928
84. Chan D et al (2003) Transformation induced by Ewing's sarcoma associated EWS/FLI-1 is suppressed by KRAB/FLI-1. Br J Cancer 88:137–145
85. Chansky HA et al (2004) Targeting of EWS/FLI-1 by RNA interference attenuates the tumor phenotype of Ewing's sarcoma cells in vitro. J Orthop Res 22:910–917
86. Kovar H et al (1996) EWS/FLI-1 antagonists induce growth inhibition of Ewing tumor cells in vitro. Cell Growth Differ 7:429–437
87. Matsumoto Y et al (2001) Downregulation and forced expression of EWS-Fli1 fusion gene results in changes in the expression of G(1)regulatory genes. Br J Cancer 84:768–775
88. Ouchida M, Ohno T, Fujimura Y, Rao VN, Reddy ES (1995) Loss of tumorigenicity of Ewing's sarcoma cells expressing antisense RNA to EWS-fusion transcripts. Oncogene 11:1049–1054
89. Prieur A, Tirode F, Cohen P, Delattre O (2004) EWS/FLI-1 silencing and gene profiling of Ewing cells reveal downstream oncogenic pathways and a crucial role for repression of insulin-like growth factor binding protein 3. Mol Cell Biol 24:7275–7283
90. Tanaka K, Iwakuma T, Harimaya K, Sato H, Iwamoto Y (1997) EWS-Fli1 antisense oligodeoxynucleotide inhibits proliferation of human Ewing's sarcoma and primitive neuroectodermal tumor cells. J Clin Invest 99:239–247
91. Toretsky JA, Connell Y, Neckers L, Bhat NK (1997) Inhibition of EWS-FLI-1 fusion protein with antisense oligodeoxynucleotides. J Neuro Oncol 31:9–16
92. Kinsey M, Smith R, Lessnick SL (2006) NR0B1 is required for the oncogenic phenotype mediated by EWS/FLI in Ewing's sarcoma. Mol Cancer Res 4:851–859
93. Kauer M et al (2009) A molecular function map of Ewing's sarcoma. PLoS One 4:e5415
94. Kinsey M, Smith R, Iyer AK, McCabe ER, Lessnick SL (2009) EWS/FLI and its downstream target NR0B1 interact directly to modulate transcription and oncogenesis in Ewing's sarcoma. Cancer Res 69:9047–9055. doi:10.1158/0008-5472.CAN-09-1540
95. Baer C et al (2004) Profiling and functional annotation of mRNA gene expression in pediatric rhabdomyosarcoma and Ewing's sarcoma. Int J Cancer 110:687–694
96. Beauchamp E et al (2009) GLI1 is a direct transcriptional target of EWS-FLI1 oncoprotein. J Biol Chem 284:9074–9082
97. Zwerner JP et al (2008) The EWS/FLI1 oncogenic transcription factor deregulates GLI1. Oncogene 27:3282–3291. doi:10.1038/sj.onc.1210991, 1210991 [pii]
98. Jaishankar S, Zhang J, Roussel MF, Baker SJ (1999) Transforming activity of EWS/FLI is not strictly dependent upon DNA-binding activity. Oncogene 18:5592–5597
99. Welford SM, Hebert SP, Deneen B, Arvand A, Denny CT (2001) DNA binding domain-independent pathways are involved in EWS/FLI1-mediated oncogenesis. J Biol Chem 276:41977–41984
100. Gangwal K et al (2008) Microsatellites as EWS/FLI response elements in Ewing's sarcoma. Proc Natl Acad Sci U S A 105:10149–10154. doi:10.1073/pnas.0801073105
101. Mao X, Miesfeldt S, Yang H, Leiden JM, Thompson CB (1994) The FLI-1 and chimeric EWS-FLI-1 oncoproteins display similar DNA binding specificities. J Biol Chem 269:18216–18222
102. Gangwal K, Lessnick SL (2008) Microsatellites are EWS/FLI response elements: genomic "junk" is EWS/FLI's treasure. Cell Cycle 7:3127–3132
103. Guillon N et al (2009) The oncogenic EWS-FLI1 protein binds in vivo GGAA microsatellite sequences with potential transcriptional activation function. PLoS One 4:e4932
104. Sankar S et al (2013) Mechanism and relevance of EWS/FLI-mediated transcriptional repression in Ewing sarcoma. Oncogene 32(42):5089–5100

105. Sorna V et al (2013) High-throughput virtual screening identifies novel N′-(1-phenylethylidene)-benzohydrazides as potent, specific, and reversible LSD1 inhibitors. J Med Chem 56:9496–9508. doi:10.1021/jm400870h
106. Prasad DD, Rao VN, Lee L, Reddy ES (1994) Differentially spliced erg-3 product functions as a transcriptional activator. Oncogene 9:669–673
107. Ginsberg JP et al (1999) EWS-FLI1 and EWS-ERG gene fusions are associated with similar clinical phenotypes in Ewing's sarcoma. J Clin Oncol 17:1809–1814
108. Braunreiter CL, Hancock JD, Coffin CM, Boucher KM, Lessnick SL (2006) Expression of EWS-ETS fusions in NIH3T3 cells reveals significant differences to Ewing's sarcoma. Cell Cycle 5:2753–2759
109. Wang L et al (2007) Undifferentiated small round cell sarcomas with rare EWS gene fusions: identification of a novel EWS-SP3 fusion and of additional cases with the EWS-ETV1 and EWS-FEV fusions. J Mol Diagn 9:498–509
110. Ng TL et al (2007) Ewing sarcoma with novel translocation t(2;16) producing an in-frame fusion of FUS and FEV. J Mol Diagn 9:459–463
111. Shing DC et al (2003) FUS/ERG gene fusions in Ewing's tumors. Cancer Res 63:4568–4576
112. Chansky HA, Hu M, Hickstein DD, Yang L (2001) Oncogenic TLS/ERG and EWS/Fli-1 fusion proteins inhibit RNA splicing mediated by YB-1 protein. Cancer Res 61:3586–3590
113. Cironi L et al (2008) IGF1 is a common target gene of Ewing's sarcoma fusion proteins in mesenchymal progenitor cells. PLoS One 3:e2634
114. Rhodes M et al (1998) A high-resolution microsatellite map of the mouse genome. Genome Res 8:531–542
115. Kovar H et al (1990) Overexpression of the pseudoautosomal gene MIC2 in Ewing's sarcoma and peripheral primitive neuroectodermal tumor. Oncogene 5:1067–1070
116. Szuhai K et al (2009) The NFATc2 gene is involved in a novel cloned translocation in a Ewing sarcoma variant that couples its function in immunology to oncology. Clin Cancer Res 15:2259–2268
117. Chen L, Glover JN, Hogan PG, Rao A, Harrison SC (1998) Structure of the DNA-binding domains from NFAT. Fos and Jun bound specifically to DNA. Nature 392:42–48. doi:10.1038/32100
118. Macian F (2005) NFAT proteins: key regulators of T-cell development and function. Nat Rev Immunol 5:472–484. doi:10.1038/nri1632
119. Macian F, Lopez-Rodriguez C, Rao A (2001) Partners in transcription: NFAT and AP-1. Oncogene 20:2476–2489. doi:10.1038/sj.onc.1204386
120. Rao A, Luo C, Hogan PG (1997) Transcription factors of the NFAT family: regulation and function. Annu Rev Immunol 15:707–747. doi:10.1146/annurev.immunol.15.1.707
121. Kim S, Denny CT, Wisdom R (2006) Cooperative DNA binding with AP-1 proteins is required for transformation by EWS-ETS fusion proteins. Mol Cell Biol 26:2467–2478
122. Thomas RS et al (1997) ETS1, NFkappaB and AP1 synergistically transactivate the human GM-CSF promoter. Oncogene 14:2845–2855. doi:10.1038/sj.onc.1201125
123. Verger A et al (2001) Identification of amino acid residues in the ETS transcription factor Erg that mediate Erg-Jun/Fos-DNA ternary complex formation. J Biol Chem 276:17181–17189
124. Yamaguchi S et al (2005) EWSR1 is fused to POU5F1 in a bone tumor with translocation t(6;22)(p21;q12). Gene Chromosome Cancer 43:217–222
125. Nichols J et al (1998) Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell 95:379–391
126. Okamoto K et al (1990) A novel octamer binding transcription factor is differentially expressed in mouse embryonic cells. Cell 60:461–472
127. Rosner MH et al (1990) A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. Nature 345:686–692. doi:10.1038/345686a0
128. Mastrangelo T et al (2000) A novel zinc finger gene is fused to EWS in small round cell tumor. Oncogene 19:3799–3804

129. Bossone SA, Asselin C, Patel AJ, Marcu KB (1992) MAZ, a zinc finger protein, binds to c-MYC and C2 gene sequences regulating transcriptional initiation and termination. Proc Natl Acad Sci U S A 89:7452–7456
130. Bochar DA et al (2000) A family of chromatin remodeling factors related to Williams syndrome transcription factor. Proc Natl Acad Sci U S A 97:1038–1043
131. Bozhenok L, Wade PA, Varga-Weisz P (2002) WSTF-ISWI chromatin remodeling complex targets heterochromatic replication foci. EMBO J 21:2231–2241. doi:10.1093/emboj/21.9.2231
132. LeRoy G, Loyola A, Lane WS, Reinberg D (2000) Purification and characterization of a human factor that assembles and remodels chromatin. J Biol Chem 275:14787–14790. doi:10.1074/jbc.C000093200
133. Percipalle P, Farrants AK (2006) Chromatin remodelling and transcription: be-WICHed by nuclear myosin 1. Curr Opin Cell Biol 18:267–274. doi:10.1016/j.ceb.2006.03.001
134. Strohner R et al (2001) NoRC – a novel member of mammalian ISWI-containing chromatin remodeling machines. EMBO J 20:4892–4900. doi:10.1093/emboj/20.17.4892
135. Sumegi J et al (2011) A novel t(4;22)(q31;q12) produces an EWSR1-SMARCA5 fusion in extraskeletal Ewing sarcoma/primitive neuroectodermal tumor. Mod Pathol Off J U S Can Acad Pathol 24:333–342. doi:10.1038/modpathol.2010.201
136. Machado I et al (2013) Superficial EWSR1-negative undifferentiated small round cell sarcoma with CIC/DUX4 gene fusion: a new variant of Ewing-like tumors with locoregional lymph node metastasis. Virchows Arch Int J Pathol 463:837–842. doi:10.1007/s00428-013-1499-9
137. Pierron G et al (2012) A new subtype of bone sarcoma defined by BCOR-CCNB3 gene fusion. Nat Genet 44:461–466. doi:10.1038/ng.1107
138. de Alava E, Lessnick SL, Sorensen PH (2013) In: Fletcher CDM, Bridge JA, Hogendoorn PCW, Mertens F (eds) WHO classification of tumours of soft tissue and bone. International Agency for Research on Cancer (IARC), Lyons, France, pp 305–309, Ch. 19
139. Carpentieri DF et al (2005) Protocol for the examination of specimens from pediatric and adult patients with osseous and extraosseous ewing sarcoma family of tumors, including peripheral primitive neuroectodermal tumor and ewing sarcoma. Arch Pathol Lab Med 129:866–873
140. Folpe AL et al (2005) Morphologic and immunophenotypic diversity in Ewing family tumors: a study of 66 genetically confirmed cases. Am J Surg Pathol 29:1025–1033
141. Bennani-Baiti IM, Machado I, Llombart-Bosch A, Kovar H (2012) Lysine-specific demethylase 1 (LSD1/KDM1A/AOF2/BHC110) is expressed and is an epigenetic drug target in chondrosarcoma, Ewing's sarcoma, osteosarcoma, and rhabdomyosarcoma. Hum Pathol 43:1300–1307. doi:10.1016/j.humpath.2011.10.010

Part V
Epithelial Tumours

Chapter 16
RET and Thyroid Carcinomas

Maria Domenica Castellone and Giancarlo Vecchio

Contents

16.1 Thyroid Carcinomas 358
16.2 Role of RET Tyrosine Kinase in Human Thyroid Carcinomas 360
16.2.1 Structure of RET 360
16.2.2 RET Rearrangements in Thyroid Papillary Carcinoma 361
16.2.3 Signal Transduction Mediated by Normal and Oncogenic RET Tyrosine Kinase 363
16.3 Effect of Radiation in the Pathogenesis of Human Thyroid Carcinomas 365
16.3.1 Role of Radiations in Thyroid Carcinogenesis 365
16.3.2 The Chernobyl Accident 365
16.3.3 Molecular Studies on Thyroid Carcinomas Following External and Internal Radiation 366
16.4 RET Rearrangements in Cancers Other than Thyroid 369
16.4.1 Leukaemia 369
16.4.2 Lung Cancer 371
16.4.3 Other Cancers 371
16.5 Diagnostic and Therapeutic Role of *RET/PTC* Rearrangements 371
Acknowledgements 372
References 372

Abstract Papillary Thyroid Carcinoma (PTC) is the most common thyroid malignancy. The molecular basis of thyroid carcinogenesis has been widely investigated in the past years. Approximately 70 % PTCs features BRAF serine/threonine kinase point mutation or rearrangement of the RET tyrosine-kinase receptor through intrachromosomal inversions or inter-chromosomal translocations. After the Chernobyl accident, the incidence of childhood PTCs increased drastically. This dependence on specific genetic lesions has highlighted thyroid cancer as an ideal target for

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molecular inhibition. Recent studies have identified small-molecule ATP-competitive of RET protein kinase inhibitors (PKI) as effective at suppressing proliferation of thyroid cancer cells.

Keywords Thyroid carcinoma • RET • Tyrosine kinase • Signal transduction • Radiations

16.1 Thyroid Carcinomas

The thyroid gland is located on the anterior surface of the trachea at the basis of the neck. It is composed of two different cell types: follicular and parafollicular cells. Follicular cells are epithelial cells organised in spherical structures called follicles. They have the unique property of concentrating iodide, introduced through the diet, by an active transport mechanism and incorporate it into the protein thyroglobulin, to form iodotyrosines, the precursors of L-tri-iodothyronine (T3) and L-thyroxine (T4) (thyroid hormones) which are released into the circulation under the control of the hypothalamic-pituitary axis. Parafollicular cells or C-cells, originate from the neural crest, are scattered in the interfollicular space and are responsible for the production of the calcium-regulating hormone calcitonin.

Thyroid neoplasms can derive from follicular cells or from C-cells. Follicular cell-derived lesions are broadly subdivided in benign and malignant [1]. Benign thyroid lesions are typically solitary adenomas and are extremely common (up to 10 % of the general population) [2]. They can be clinically silent or hyperfunctioning (referred to as toxic thyroid adenomas), thereby causing hyperthyroidism with increased levels of thyroid hormones. Gain-of-function mutations of TSHR (~80 %) and GNAS, encoding GSα, (~20 %) are the most common genetic lesions in functional thyroid adenomas, whereas these genetic lesions do not occur in thyroid malignancies [3].

Malignant thyroid lesions are the most common cancers of endocrine organs and represent approximately 1 % of newly diagnosed cancer cases [4, 5], with an incidence that is rising more rapidly than any other cancer in the U.S., tripling in the last decade [6]. It is now the eighth most incidental cancer overall and the sixth most incidental cancer in women [6]. Although this may be partly attributable to the improved diagnostic methodologies, the incidence of large thyroid cancers (>5 cm) is also increasing, moreover population adjusted disease specific mortality has also increased more than 30 % in the last 10 years [6]. Cancers deriving from follicular cells represent more than 95 % of thyroid cancers whereas only 3–5 % of them arise from C-cells. On the basis of histological and clinical parameters, malignant follicular-derived lesions are classified into well differentiated

carcinomas (WDC), poorly differentiated carcinomas (PDC), and anaplastic carcinomas (ATC, 2 %) [2, 7–9]. Well-differentiated carcinomas represent more than 90 % of thyroid cancer cases and are divided in papillary (PTC, 85 %) and follicular (FTC, 5–10 %) thyroid carcinomas [10]. PTC is the most common thyroid malignancy. It features a distinctive architectural pattern characterised by a branching (papillary) architecture and a peculiar nuclear morphology. PTC is generally known to display varying histological structures and therefore it is further subdivided into subtypes or variants. According to the WHO histological classification, these variants include classic papillary, follicular, macrofollicular, solid, oxyphilic-cell, clear-cell, diffuse-sclerosing, tall-cell, columnar-cell, cribriform-morular, and Warthin-like variants. Papillary microcarcinoma is also considered to be a separate variant. The solid, tall-cell and diffuse-sclerosing variants of PTC have been associated with increased malignancy [11–15]. PTC is more frequent in women than men and affects patients 20–50 years old. It can also occur in childhood as consequence of accidental or therapeutic radiation exposure. PTC has an indolent behaviour, tendency to form metastasis to local lymph nodes and a survival rate greater than 90 % [9, 11, 16].

FTC is less frequent than PTC; pre-operative differentiation of FTC from benign adenomas is often a difficult task. Well differentiated thyroid cancers are generally still capable of concentrating iodide, a feature greatly useful for the therapeutic treatment of these tumours and of their metastases with radioactive iodide. PTC and, to a less extent, FTC have a good prognosis if properly treated [17], with a predicted death rate over 30–50 years of 1 %. Despite this, there is a recurrence rate of 30 % [18]. However, both PTC and FTC may sometimes show an aggressive behaviour and lose the ability to concentrate radioiodine. These types of thyroid cancers are referred to as Progressive Metastatic Radioiodine Refractory (PMRR) differentiated thyroid cancers. ATC is one of the most aggressive solid tumours in humans. It is rapidly fatal, with a mean survival of 6 months after diagnosis [19]. Finally, medullary thyroid carcinoma (MTC) is a rare tumour that arises from thyroid parafollicular C cells. Together with pheochromocytoma, parathyroid adenoma and other tumour types, MTC can be inherited in the context of autosomal dominant MEN 2 (multiple endocrine neoplasia type 2) syndromes (MEN 2A, MEN 2B and FMTC) [20].

At least in some cases, thyroid carcinogenesis may be explained by a multi-step model (Fig. 16.1). According to this model, well-differentiated tumours (PTC and FTC) occur after early initiating events such as *RET/PTC* rearrangements and *BRAF* mutations or RAS gene mutations and *PAX8-PPARG* rearrangement [21], whereas additional mutations of genes like *TP53*, *CTNNB1* and *PIK3CA* lead to ATC [22–24]. *CCND1* overexpression or *CDKN1B/p27* downregulation are implicated particularly in aggressive WDC subtypes [2, 7, 25] (Fig. 16.1).

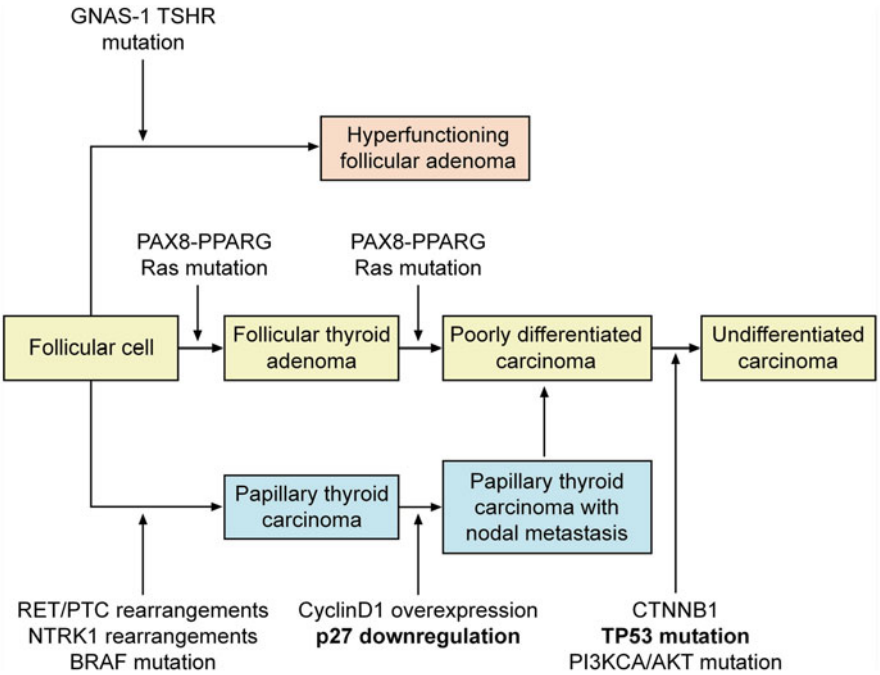

Fig. 16.1 Model of thyroid multi-step carcinogenesis. The genetic lesions associated to thyroid carcinomas can affect either proto-oncogenes (gain-of-function mutations) or tumour suppressor genes (loss-of-function mutations) (*bold characters*)

16.2 Role of RET Tyrosine Kinase in Human Thyroid Carcinomas

16.2.1 Structure of RET

The RET oncogene is located on 10q11.2 and encodes a transmembrane receptor tyrosine kinase (RTK) with four cadherin-related motifs in the extracellular domain [26]. *RET* is normally expressed in the developing central and peripheral nervous system and is an essential component of a signalling pathway required for renal organogenesis and enteric neurogenesis. It is normally expressed at high levels in C-cells, but not in follicular cells [27]. Glial cell line-derived neurotrophic factor (GDNF)-family ligands and GDNF-family receptor α (GFRA1) bind the extracellular domain of RET [28, 29]. Upon binding to ligand, RET dimerises and autophosphorylates various cytoplasmic tyrosines within its intracellular domain. The phosphorylated tyrosines thus become binding sites for intracellular molecules containing phosphotyrosine-binding motifs (SH2 or PTB), thereby initiating a diverse array of signalling pathways [26, 27].

16.2.2 RET Rearrangements in Thyroid Papillary Carcinoma

The first evidence of *RET* involvement in human cancer was obtained in thyroid gland papillary carcinomas [30]. Three years later, the oncogene was molecularly cloned: it was demonstrated that it was a chimeric gene generated by the fusion of the 3′ *RET* sequences encoding the tyrosine kinase domain with the 5′ terminal region of a new gene denominated CCDC6 (formerly called H4). This oncogene was named *RET/PTC* [31]. The chimeric gene was generated by chromosomal inversion occurring on chromosome 10, both genes, *RET* and *CCDC6* lying on the same chromosome. Following this first rearrangement occurring in PTC's several other chromosomal aberrations involving the *RET* gene have been described. Such chromosomal aberrations all give rise to chimeric *RET/PTC* oncogenes, either by chromosomal inversion or translocation. Rearrangements have been shown to be restricted to papillary type of thyroid cancer [32, 33]. *RET/PTC* oncogenes are composed of the tyrosine kinase and COOH tail encoding sequence of *RET* (from exon 12 to the 3′ end) and fused to the 5′ end of the promoter sequence and 5′ terminal exons of heterologous genes possessing protein-protein interaction motifs [34] (Fig. 16.2). Intra-chromosomal inversions or inter-chromosomal translocations of 10q11.2 occur in 2.5–40 % of PTC's. This provides RET/PTC kinases with dimerising interfaces, thereby resulting in ligand-independent autophosphorylation [7]. RET/PTC proteins lack the *RET* signal peptide and transmembrane domains but retain the kinase domain and most of the autophosphorylation sites, thereby allowing downstream signalling [29]. By providing an active transcriptional promoter, *RET/PTC* rearrangements enable thyroid expression of the chimeric RET/PTC oncoproteins. To date, 17 *RET/PTC* rearrangements have been described; the breakpoint for rearrangement are all located in intron 11 of the *RET* gene (Table 16.1). The most frequent are *RET/PTC1*, which involves *RET* and *CCDC6* [30, 31, 35] and *RET/PTC3*, between *RET* and *NCOA4* (Ret-Fused Gene) (also named *RFG*, *ELE1* or *ARA70*) [36, 37] (Fig. 16.2 and Table 16.1). These oncoproteins induce transformation and de-differentiation of cultured thyroid cells [38, 39], and thyroid-targeted expression of *RET/PTC1* or *RET/PTC3* induces thyroid neoplasms in mice [40].

A body of evidence suggests the idea that the *RET/PTC* oncogenes can be causative in thyroid tumorigenesis and also may represent an early genetic change in PTC development. The reported *RET/PTC* prevalence in thyroid tumours varies greatly in different reports [10, 41]. The method to detect *RET/PTC* rearrangement is important and it therefore reflects on the reported prevalence [42]. *RET/PTC* rearrangements have been found also in 10–45 % of thyroid adenomas and other non neoplastic lesions and in Hashimoto's thyroiditis [43–49]. In most populations, *RET/PTC1* is the most common type of *RET/PTC* as it comprises 60–70 % of positive cases, whereas *RET/PTC3* accounts for 20–30 % and *RET/PTC2* and other novel rearrangement types for less than 5–10 % [50, 51]. *RET/PTC1* is more frequently associated with classic PTC and with the diffuse sclerosing variant PTC; conversely, *RET/PTC3* is more common in the solid variant and in PTC associated to ionizing radiation exposure [52] (see below Sect. 16.3.3). Moreover, at

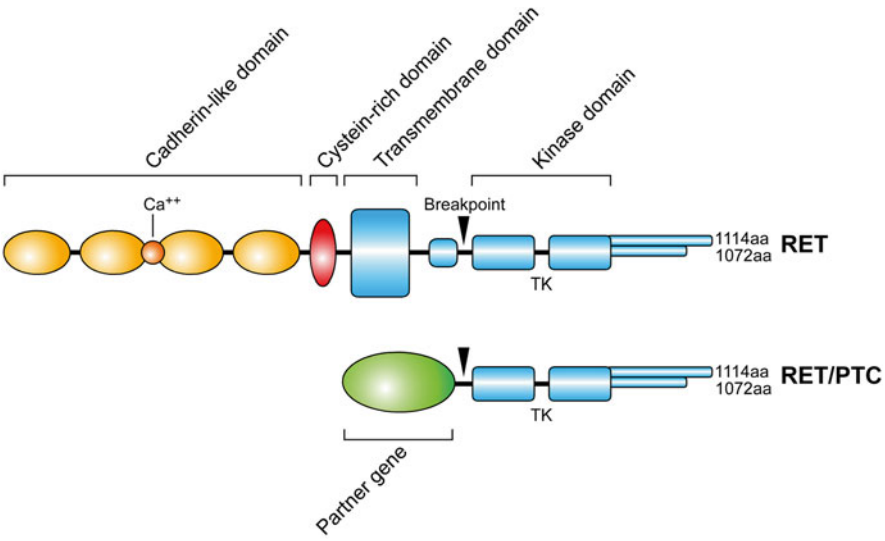

Fig. 16.2 Structure of RET and RET/PTC rearranged proteins

Table 16.1 RET/PTC rearrangements in thyroid tumors

Oncogene	Donor gene	Chromosomal rearrangements
RET/PTC1	*CCDC6*	inv(10)(q11.2q21.2)
RET/PTC2	*PRKAR1A*	t(10;17)(q11.2;q23)
RET/PTC3	*NCOA4*	inv(10)(q11.2q11.2)
RET/PTC4	*NCOA4*	inv(10)(q11.2q11.2)
RET/PTC5	*Golgas*	t(10;14)(q11.2;q32)
RET/PTC6	*TRIM24*	t(7;10)(q34;q11.2)
RET/PTC7	*TRIM33*	t(1;10)(p13.2;q11.2)
RET/PTC8	*KTN1*	t(10;14)(q11.2;q22.3)
RET/PTC9	*RFG9*	t(10;18)(q11.2;q21–22)
ELKS/RET	*ERC1/ELKS*	t(10;12)(q11.2;p13.3)
PCM1/RET	*PCM1*	t(8;10)(p22;q11.2)
RFP/RET	*TRIM27*	t(6;10)(p22.1;q11.2)
HOOK3/ RET	*HOOK3*	t(8;10)(p11.21;q11.2)

variance from *BRAF* (see below), *RET/PTC* is not a negative prognostic factor for PTC [14]. So far no consensus concerning the clinical prognostic value of the presence of a *RET/PTC* rearrangement, either *RET/PTC1* or *RET/PTC3*, has been reached [41]. Some evidences exist suggesting that PTCs with *RET/PTC1* rearrangements are associated with a more favourable behaviour [53], while those harbouring a *RET/PTC3* rearrangement may be more aggressive [54, 55]. *RET/PTC* oncogenes are detected with a high frequency in clinically-silent small PTC,

confirming that they can be early events in thyroid tumorigenesis [21]. Moreover, the evidence that *RET*/*PTC*-transgenics develop PTC proves that *RET*/*PTC* oncogenes are able to initiate thyroid carcinogenesis [40]. On the other hand, the low prevalence of expression of RET/PTC oncoproteins in PDC and ATC suggests that they do not play a prominent role in thyroid tumour progression [32, 51].

Interestingly, most of the *RET* partner genes are represented by genes with a tumour suppressor function, suggesting how *RET*/*PTC* rearrangements might cause a genetic double hit, inducing simultaneously the gain of RET oncogenic activity and the knockdown of the *RET* partner gene. CCDC6, involved in *RET*/*PTC1* rearrangement, is a ubiquitously expressed 65 kDa protein that displays proapoptotic activity and is involved in ATM-mediated cellular response to DNA damage [56, 57]. The PRKAR1A gene, involved in *RET*/*PTC2* rearrangement, encodes for a bona fide tumour suppressor gene [58, 59]. *NCOA4*, involved in *RET*/*PTC3* variant, encodes a 70 kDa protein that when overexpressed in prostate cancer cells reduces cell proliferation and whose expression is reduced in aggressive prostate and breast cancers, thus suggesting that this gene may function as a suppressor of tumorigenesis [60–62]. *TRIM24* (also known as HTIF1), the *RET* fusion partner in *RET*/*PTC6* [63] shows frequent genetic aberrations in human hepatic cell carcinoma, strongly suggesting its role as a tumour suppressor gene [64, 65] (Table 16.1).

In addition to *RET*/*PTC*, chromosomal rearrangements involving *NTRK1* and *BRAF* genes, as well as other gene rearrangements, also occur in papillary thyroid carcinomas, although with a significantly lower prevalence [66, 67]. As a result, papillary thyroid carcinoma represents a good model to study the mechanisms of chromosomal rearrangements in solid tumours [68].

16.2.3 Signal Transduction Mediated by Normal and Oncogenic RET Tyrosine Kinase

As a member of the tyrosine kinases receptor family (RTK), and thanks to its intrinsic kinase activity, RET activates many intracellular signalling pathways. Upon binding to ligand, it dimerises and autophosphorylates various cytoplasmic tyrosines. The phosphorylated tyrosines thus become binding sites for intracellular molecules containing phosphotyrosine-binding motifs, thereby initiating several signalling pathways [26]. The RET intracellular domain contains at least 12 autophosphorylation sites, 11 of which are maintained in RET/PTC proteins [69]. Tyrosines 900 and 905 (Y900, Y905) map in the kinase A-loop. These tyrosines are essential for RET activation in intact cells [70], although recent biochemical data showed that conformational change of the A-loop is not essential for RET kinase activation [71]. Y905 acts also as a binding site for GRB7 and GRB10 adaptors [72] and mediates binding of SH2B1b, a protein that, by obstructing the NSFL1C/SHP1 tyrosine phosphatase, enhances RET phosphorylation [73].

Y1015, a docking site for phospholipase Cγ [74], is essential for RET function during kidney development probably by SPRY (Sprouty) gene activation [75], and for RET/PTC transforming activity [74]. Y981 is a binding site for SRC [76]. Y1062 is essential for RET transforming activity [77–79], and for development [75, 80, 81]. Y1062 acts as a binding site for several proteins, among which SHC1P2/SHC, IRS1, IRS2, FRS2, DOK1, DOK4, DOK5, and PDLIM7/Enigma, which in turn lead to stimulation of the RAS/MAPK and phosphatidylinositol-3- kinase (PIK3CA)/AKT1 pathway [82]. Binding to SHC1P2/SHC and FRS2 mediates recruitment of GRB2-HMCES/SOS complexes so leading to GTP exchange on RAS and RAS/ERK stimulation [83, 84] (Fig. 16.3). Through Y1062, RET medi-

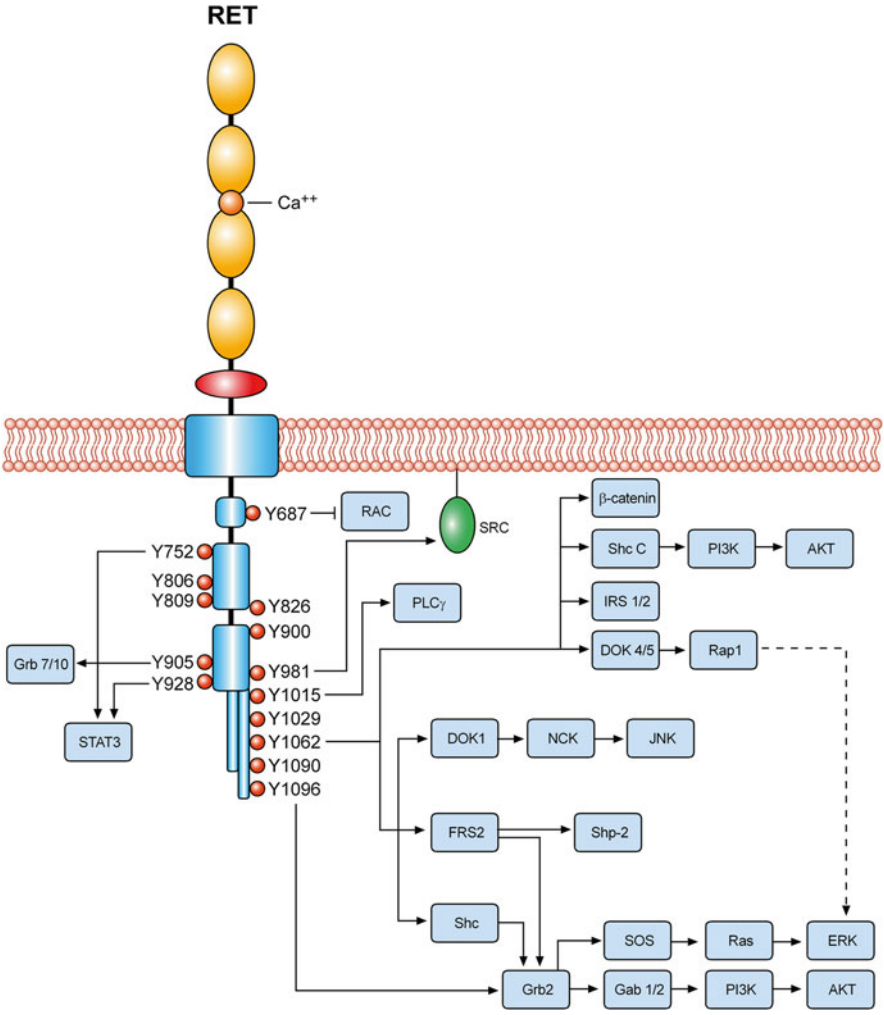

Fig. 16.3 Signalling pathways activated by RET

ates also activation of the small GTPase RAP1GAP [85]. The DOK4 adaptor plays a role in RET-mediated RAP1GAP activation in pheochromocytoma PC12 cells [86]. Both RAP1GAP and DOK4 contribute to MAPK activation [85, 86] (Fig. 16.3).

RET/PTC signals along the ERK pathway [78, 87, 88], but other pathways, particularly the PIK3CA/AKT1 one, may contribute to its biological effects as well [89–92]. RET/PTC-mediated activation of PIK3CA/AKT1 and RAS/ERK also contributes to induce tyrosine phosphorylation of CTNNB1, thereby mobilizing the fraction associated to CDH1 (E-cadherin) and increasing its free cytosolic and nuclear pool [93] (Fig. 16.3).

Finally, the activity of RET is controlled at multiple levels by transcription factors that regulate receptor and ligand expression [94–96]; by extracellular signalling factors, such as SRGAP1 (Robo/Slit) [97] and BMP4 [94, 98]; and by intracellular inhibitors of tyrosine kinase activity like SPRY proteins [99, 100] or PTEN, whose expression can suppress GDNF/RET-mediated chemotaxis and kidney development [101].

16.3 Effect of Radiation in the Pathogenesis of Human Thyroid Carcinomas

16.3.1 Role of Radiations in Thyroid Carcinogenesis

In children, whose thyroid glands are proliferating rapidly [102] or in adult animals with glands stimulated by goitrogens [103], the cells injured by radiation and with abnormal growth potential are able to develop into gross neoplastic lesions. In 1950 the first epidemiologic study relating external beam radiation exposure and childhood thyroid cancer was published [104]. In Hiroshima and Nagasaki well over 100,000 people were exposed to external whole-body radiation from gamma rays and neutrons. Exposure to fallout from the radioactive isotopes released from the explosion was trivial [105]. The first study concerning the increase of thyroid cancer as a consequence of the Japan bombing was reported by Wood [106]. Conard and colleagues reported in 1970 an increased incidence of thyroid tumours in inhabitants of regions affected by a thermonuclear test [107] and in 1985 another research group confirmed the increase in thyroid cancer in patients with a history of external radiation for benign or malignant conditions [108]. All these studies underline the effects of external radiation in the pathogenesis of childhood thyroid cancer.

16.3.2 The Chernobyl Accident

The release of radioiodine in the aftermath of the Chernobyl nuclear plant accident in April 1986 led to an unprecedented exposure of the thyroid glands of inhabitants of Belarus, Ukraine and Russia. After Chernobyl, millions of people were exposed to significant levels of radioactivity from fallout. External whole body radiation was

relevant only to those working in or close to the reactor. The radiation from the released isotopes was largely beta and gamma radiation. While the whole body radiation from the atomic bombs affected all organs fairly evenly, the isotopic radiation from Chernobyl affected organs differently depending on the nature of the isotope and the dose from the fallout was influenced by many factors: time, atmospheric, dietary and environmental [109]. Thyroid cancer in young people is a rare disease, with an incidence of around 0.5–1.5 cases per million per year [110]. As early as 4 years after the accident, the incidence of childhood PTCs in the most contaminated region, had increased drastically, exceeding 100 cases per million children per year [111–113].

16.3.3 Molecular Studies on Thyroid Carcinomas Following External and Internal Radiation

Ionizing-radiations associated PTCs have a high prevalence of *RET*/*PTC* rearrangements and this genetic lesion has been mechanistically linked to radiation exposure. This finding is in agreement with results of experiments showing that in vitro irradiation is able to originate *RET* rearrangements in cultured human thyroid cells [114, 115], as well as in human foetal thyroid tissue xenografts in SCID mice [116, 117]. The generation of *RET*/*PTC* rearrangements [68, 118], as well as of another rearrangement which can occur in papillary thyroid cancer, i.e., the *TPR*/*NTRK1* rearrangement [119] have also been demonstrated in normal thyroid cultured cells after in vitro exposure to radiation. By using the human thyroid cell line Nthy-ori 3-1, which had been found to harbour *RET*/*PTC1* after gamma radiation, it has been demonstrated that H_2O_2, generated during a 5Gy X-ray irradiation, causes DNA double strand breaks and contributes to *RET*/*PTC1* formation [120]; therefore, the oxidative stress derived from H_2O_2 production could be responsible for occurrence of *RET*/*PTC1* rearrangement [120]. These studies provide evidence for the direct link between exposure to ionizing radiation and generation of *RET*/*PTC* rearrangement in human thyroid cells [118]. *RET* partner genes *CCDC6* and *NCOA4* are located in common fragile sites FRA10C and FRA10G and undergo DNA breakage after exposure to fragile site break-inducing chemicals [118] and, therefore, can undergo DNA double strand breaks upon exposure to ionizing radiation. Ionizing radiation damages DNA in a variety of ways as a result of either direct energy deposition along the radiation track or by secondary reactive oxygen species produced by ionisation of water [118]. Moreover, in the interphase chromatin of thyroid cells, *CCDC6* and *RET* genes frequently overlap. This spatial proximity facilitates genetic recombination, giving rise to the CCDC6-RET (RET/PTC1) oncoprotein [68]. This recombination event was first shown for *RET* and *CCDC6* in a study that utilised fluorescence in situ hybridisation (FISH) and three dimensional (3D) confocal microscopy and showed that these genes were non randomly located with respect to each other in the interphase nuclei of thyroid cells and were much closer than

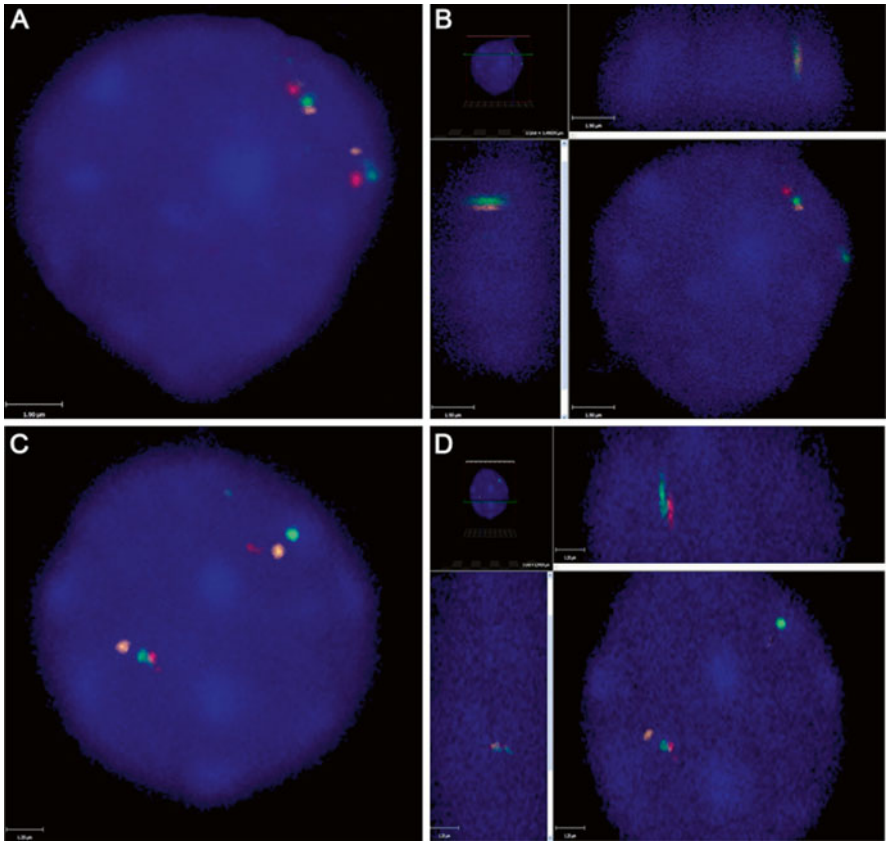

Fig. 16.4 Three-colour fluorescence in situ hybridisation (*FISH*) showing positioning of *RET* (*green*), *NCOA4* (*orange*) and *HINFP*/*H4* (*red*) in interphase nuclei of thyroid cells. (a) 2D image of a nucleus showing two sets of *RET*, *NCOA4* and *HINFP*/*H4* signals with one *RET* and one *NCOA4* signal positioned close to each other. (**b**) 3D image showing that *RET* and *NCOA4* are juxtaposed to each other in the same z plane. (**c**) 2D image of a nucleus showing one *RET* signal and one *HINFP*/*H4 signal* positioned close to each other. (**d**) 3D image showing that *RET* and *HINFP*/*H4* signals are juxtaposed to each other in the same z plane. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article reported in ref. 69) (Figure reprinted from Gandhi et al. [68]. With permission from Elsevier)

expected, based on their genomic separation [38, 68] (Fig. 16.4). This study also showed that the proximity between potentially recombinogenic genes was cell type specific and was not present in some non thyroid cells such as mammary epithelial cells. Similar findings have been obtained for *RET* and *NCOA4*, the partners of the *RET*/*PTC3* rearrangement [68]. In addition, spatial proximity was found to exist between the partners of another rearrangement occurring in papillary thyroid cancer – *NTRK1* was shown to be closer to its translocation partner, *TPR*, in thyroid cells but not in lymphocytes [119]. Propensity of thyrocytes to increase DNA

end-joining activity upon radiation-induced DNA damage may further facilitate the occurrence of *RET*/*PTC* chromosomal rearrangements [68, 121, 122].

Several studies have tried to correlate the pathological structure of the tumours found in the area of Chernobyl with the molecular alterations found in the same tumours. Initial studies carried out on Belarusian cohorts [123–125], suggested an increased frequency of *RET*/*PTC* rearrangements in radiation induced PTCs in children. In a study on Ukrainian cases carried on 106 papillary carcinomas [126], 20 cases positive for *RET*/*PTC1*, 15 for *RET*/*PTC3* and 1 positive for both rearrangements were found. Essentially similar results were obtained examining different Belarusian and Ukrainian cohorts by Thomas and colleagues [52]. In this study the type of *RET*/*PTC* rearrangement correlated with the tumour morphology; *RET*/*PTC3* dominated in the solid subtype and *RET*/*PTC1* was more common in the classical subtype of PTC, in agreement with previous studies on other Chernobyl-related tumour cohorts [124, 125, 127].

A recent study conducted on 62 PTCs diagnosed in a Ukrainian cohort of patients who were less than 18 years old in 1986 and received 0.008–8.6 Gy of I-131 to the thyroid, showed that *RET*/*PTC* rearrangements were most common (35 %), followed by *BRAF* (15 %) and *RAS* gene (8 %) point mutations. Two tumours carrying *PAX8*/*PPARG* rearrangements were also identified. These results provide the first demonstration of *PAX8*/*PPARG* rearrangements (a rearrangement previously associated only with follicular thyroid carcinomas and adenomas) in post Chernobyl tumours and support the relationship between chromosomal rearrangement, but not point mutations, and I-131 exposure [128]. In order to establish the exact role of radiation in generating fusion oncoproteins a large cohort of post-Chernobyl cancer specimens were analysed in a recent study by Ricarte-Filho and collaborators [129], to identify fusion oncogenes possibly acting as driver translocations in this disease. The authors demonstrated that 22 out of 26 Ukrainian patients with thyroid cancer, who were younger than 10 years of age and living in contaminated areas during the time of the Chernobyl accident, harboured fusion oncogenes that arose primarily through intrachromosomal rearrangements. Fusion oncogenes, instead, were less prevalent in tumours from a cohort of children with paediatric cancers that had not been exposed to radiation but were from the same geographical regions.

In particular, they first screened the tumours by a candidate gene approach for known oncogenic events: this analysis showed that 69 % of the patients examined had known rearrangements (15 *RET*/*PTC*, of which 6 *RET*/*PTC3*, 4 *RET*/*PTC1*, 2 *RET*/*PTC6*, 1 *RET*/*PTC* delta-3, 1 *RET*/*PTC9* and 1 *RET*/*PTC* with unknown partner; 1 *TPR-NTRK1* (*TRK2*); 1 *PAX8-PPARG* and 1 *AKAP9-BRAF*). Five radiation-exposed tumours which presented no driver alteration by the candidate gene approach were subjected to further analysis for paired-end RNA sequencing. In four out of five samples the authors were able to identify novel somatic fusions with likely oncogenic properties: one with *CREB3L2-PPARG*, one with acylglycerol kinase-BRAF (*AGK-BRAF*) and two with ETS variant 6- neurotrophic tyrosine kinase receptor, type 3 (*ETV6-NTRK3*). This study provides a clear paradigm of tumorigenesis driven by fusion oncogenes that activate MAPK signalling in thyroid papillary cancers [129].

A study conducted on thyroid carcinomas from patients who had received external radiation for benign or malignant conditions showed that, in analogy with the Chernobyl cancers, the overall frequency of RET rearrangements in papillary carcinomas, was high (84 %). But, in contrast with the results obtained in some of the studies carried out in Chernobyl tumours, the most frequently observed chimeric gene was *RET/PTC1* instead of *RET/PTC3* [130]. *RET* rearrangements have also been found in thyroid carcinoma tissue samples obtained from operations at the Semipalatinsk regional hospitals collected between 1986 and 1996, which developed around the region of Semipalatinsk nuclear testing site in Kazkhstan, in the former USSR. Out of 33 tissue blocks examined, 16 showed overexpression of *RET* mRNA. Of the 16 samples 8 were examined for *RET/PTC* rearrangement. Rearrangements of *RET/PTC3* were found in two out of eight samples (25 %), whereas no *RET/PTC1* rearrangement was found [131]. To clarify which gene alteration, chromosomal aberration, or point mutation preferentially occurs in radiation-associated adult-onset papillary thyroid cancer, molecular analyses on *RET/PTC* rearrangements and *BRAF* V600E mutation in 71 PTC cases among atomic bomb survivors, in relation to radiation dose as well as time elapsed since atomic bomb radiation exposure, have been carried out by Hamatani and colleagues [132]. *RET/PTC* rearrangements showed significantly increased frequency with increased radiation dose. In contrast, *BRAF* V600E mutation was less frequent in cases exposed to higher radiation dose.

16.4 *RET* Rearrangements in Cancers Other than Thyroid

16.4.1 Leukaemia

For many years RET activation has been related only to thyroid malignancies. Recent reports have opened the possibility that this gene can instead work as an oncogene in different systems (Fig. 16.5). *RET* rearrangements causing the fusion of *RET* tyrosine kinase gene to *FGFR1OP* (fibroblast growth factor receptor 1 oncogene partner) or *BCR* genes have been recently reported in chronic myelomonocytic leukaemia (CMML) [133]. The two *RET* fusion genes generated by two balanced translocations t(10;22) and t(6;10) respectively led to aberrant activation of RET and are able to transform haematopoietic cells and drive differentiation towards monocytic/macrophage lineage [133]. *RET* fusion to *FGFR1OP* has also been very recently described in acute myeloid leukaemia (AML) secondary to primary myelofibrosis [134], and a previous report already suggested *RET* overexpression in human leukaemic cells as well as upregulation in AML with myelomonocytic differentiation [135, 136]. Thus, although with a still unknown prevalence, CMML and AML are clearly associated to *RET* activation through gene rearrangement.

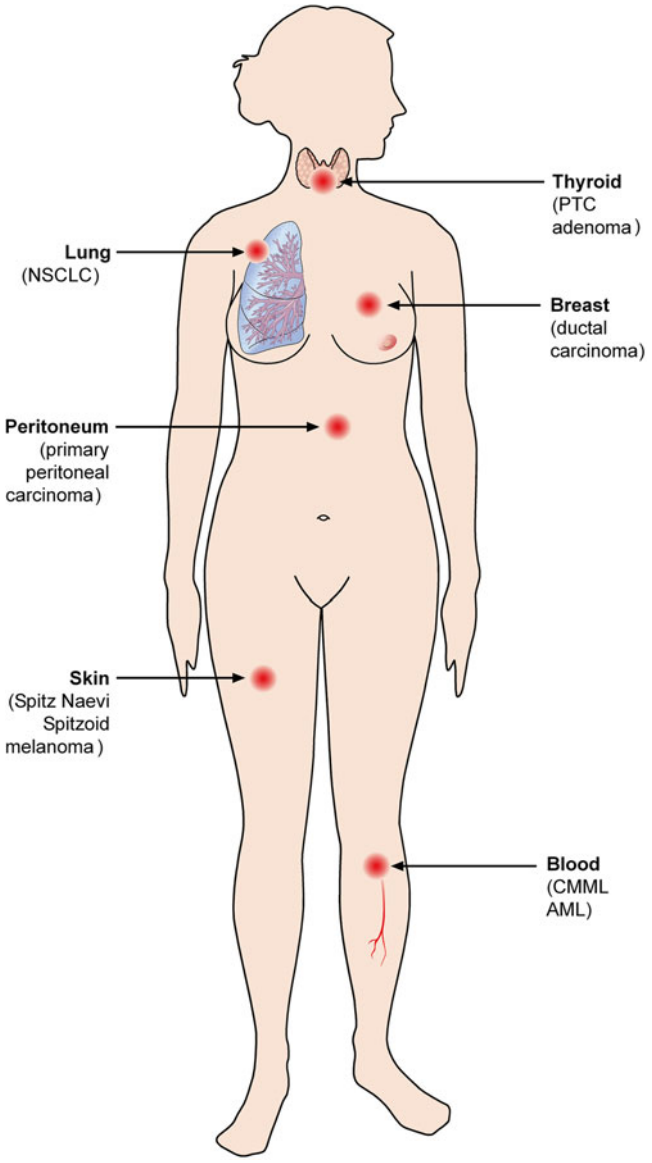

Fig. 16.5 Tumours positive for *RET* rearrangements

16.4.2 Lung Cancer

Several independent studies have identified *RET* rearrangements in a subset of lung adenocarcinomas (about 2 % of NSCLCs), predominantly occurring in never-smokers and younger patients and related to a more severe prognosis when compared to EGFR mutation positive cancers. A chromosomal inversion between the long and short arms in the centromeric region of chromosome 10 generates a novel *KIF5B* (kinesin family member 5B)-*RET* fusion gene in these patients [137]. *RET-PTC1*, *RET-PTC3* and *RET-PTC7* fusions were also identified in a smaller percentage of NSCLCs [138–140]. The mutually exclusive nature of the RET fusions and other oncogenic alterations (EGFR, RAS, ALK) suggest that the *KIF5B-RET* fusion is a driver mutation. Moreover, the tumorigenic potential of KIF5B-RET, CCDC6-RET and NCOA4-RET has been confirmed using in vitro and in vivo transformation assays [138–140]. Also in this case, the transforming capacity is linked to the constitutive ligand-independent kinase activity of RET upon fusion with a coiled-coil domain of the partner gene. Because the growth of the RET positive tumours is strongly addicted to RET activity, suppression of RET could be a potent therapeutic strategy for patients with *RET* rearrangements.

16.4.3 Other Cancers

A study on chromosomal breakpoints in radiation-transformed epithelial breast cell lines has indicated *RET* as a candidate gene. Interestingly these cells express both extra-cellular and kinase domain of the receptor, suggesting different mechanism of activation than PTC rearrangements [141]. Moreover, *RET* is overexpressed in breast cancers [142] and targeting RET improves response to therapy [143, 144] raising the possibility that *RET* rearrangements could be used as diagnostic/prognostic markers. Interestingly, 20 % of primary peritoneal carcinomas have been detected with RET/PTC1 rearrangements [145]. Finally, *RET* fusions with *KIF5B* (chromosome 10) and *GOLG5* (Chromosone 14) were also found in about 3 % of Spitz naevi and spitzoid melanomas [146]. The last rearrangement has been also described in papillary thyroid carcinomas occurring in children exposed to radioactive fallout from the Chernobyl nuclear accident [147]. The occurrence of *RET* fusions early in spitzoid neoplasms suggests that is a driver event in the pathogenesis of these tumours (Fig. 16.5).

16.5 Diagnostic and Therapeutic Role of *RET/PTC* Rearrangements

The development of new technologies such as whole genome and transcriptome sequencing that can be performed within the time necessary for making therapeutic decisions has opened the possibility to identify for each cancer its unique mutation

events in order to make the medical therapeutic decision that can be more effective. This will especially benefit those patients whose cancer does not have any known cancer driven mutations. The recent identification of *RET*/*PTC* fusion genes in malignancies other than thyroid cancers has opened the possibility to use *RET* sequencing as a diagnostic tool at least in those cancers that do not contain any known driver mutation. The identification of *RET* fusions in NSCLC patients has allowed a rapid bench to bedside process leading to the initiation of a prospective phase II clinical study for advanced cancer patients using cabozantinib, a multi-tyrosine kinase inhibitor and potent RET inhibitor. Recently published preliminary results are encouraging as they show progression-free disease in all treated patients [148].

RET targeting agents fall in two groups: small-molecules inhibitors targeting the ATP-binding site of the intracellular receptor kinase domain [149] and monoclonal antibodies that either interfere with RTK activation or target RTK-expressing cells for destruction by the immune system [150]. Monoclonal antibodies against the extracellular domain of RET have been developed with the aim of using them for the treatment of medullary thyroid carcinomas, on the surface of whose cells the RET protein is expressed and constitutively activated as a consequence of gain of function point mutations targeting either extracellular cysteines or various residues in the kinase domain [151]. Several compounds able to target the RET kinase have been developed; among them Sunitinib, Sorafenib, Vandetanib and XL-184 are in clinical studies in patients with thyroid cancer [27, 152, 153]. In particular, Vandetanib demonstrated therapeutic efficacy in a phase III trial of patients with advanced MTC and has been approved by FDA for the treatment of patients with locally advanced or metastatic MTC [154]. TKI's inhibitors are being tested also in differentiated thyroid cancer patients refractory to conventional therapy. The results of phase II–III clinical trials conducted so far are promising with a partial response ranging from 14 to 49 % and stable disease from 34 to 68 % [155]. Continuous progress in understanding cancer pathogenesis and identifying molecules involved in carcinogenesis will lead to development of novel anti-cancer drugs targeting only tumour cells while sparing normal cells that could be used as first line therapy as well as in patients that are not responsive to conventional treatments.

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References

1. Mazzaferri EL (1993) Management of a solitary thyroid nodule. N Engl J Med 328:553–559
2. Kondo T, Ezzat S, Asa SL (2006) Pathogenetic mechanisms in thyroid follicular-cell neoplasia. Nat Rev Cancer 6:292–306

3. Parma J, Van Sande J, Swillens S, Tonacchera M, Dumont J et al (1995) Somatic mutations causing constitutive activity of the thyrotropin receptor are the major cause of hyperfunctioning thyroid adenomas: identification of additional mutations activating both the cyclic adenosine 3′,5′-monophosphate and inositol phosphate-Ca2+ cascades. Mol Endocrinol 9:725–733
4. Hundahl SA, Fleming ID, Fremgen AM, Menck HR (1998) A National Cancer Data Base report on 53,856 cases of thyroid carcinoma treated in the U.S., 1985–1995. Cancer 83:2638–2648
5. Gimm O (2001) Thyroid cancer. Cancer Lett 163:143–156
6. American Cancer Society, Cancer Facts and Figures, 2013. American Cancer Society, Atlanta
7. Tallini G (2002) Molecular pathobiology of thyroid neoplasms. Endocr Pathol 13:271–288
8. DeLellis RA, Williams ED (2004) Thyroid and parathyroid tumors. In DeLellis RA, Lloyd RV, Heitz PU, Eng C (eds) Tumors of endocrine organs. World Health Organization Classification of Tumors, IARC Press: Lyon, p 51–56
9. DeLellis RA (2006) Pathology and genetics of thyroid carcinoma. J Surg Oncol 94:662–669
10. Pacini F, Schlumberger M, Dralle H, Elisei R, Smit JW, European Thyroid Cancer Taskforce et al (2006) European consensus for the management of patients with differentiated thyroid carcinoma of the follicular epithelium. Eur J Endocrinol 154:787–803
11. Sherman SI (2003) Thyroid carcinoma. Lancet 361:501–511
12. Leboulleux S, Rubino C, Baudin E, Caillou B, Hartl DM et al (2005) Prognostic factors for persistent or recurrent disease of papillary thyroid carcinoma with neck lymph node metastases and/or tumor extension beyond the thyroid capsule at initial diagnosis. J Clin Endocrinol Metab 90:5723–5729
13. Adeniran AJ, Zhu Z, Gandhi M, Steward DL, Fidler JP et al (2006) Correlation between genetic alterations and microscopic features, clinical manifestations, and prognostic characteristics of thyroid papillary carcinomas. Am J Surg Pathol 30:216–222
14. Elisei R, Ugolini C, Viola D, Lupi C, Biagini A et al (2008) BRAF(V600E) mutation and outcome of patients with papillary thyroid carcinoma: a 15-year median follow-up study. J Clin Endocrinol Metab 93:3943–3949
15. Romei C, Ciampi R, Faviana P, Agate L, Molinaro E et al (2008) BRAFV600E mutation, but not RET/PTC rearrangements, is correlated with a lower expression of both thyroperoxidase and sodium iodide symporter genes in papillary thyroid cancer. Endocr Relat Cancer 15:511–520
16. Schlumberger MJ (1998) Papillary and follicular thyroid carcinoma. N Engl J Med 338:297–306
17. Elisei R, Molinaro E, Agate L, Bottici V, Masserini L et al (2010) Are the clinical and pathological features of differentiated thyroid carcinoma really changed over the last 35 years? Study on 4187 patients from a single Italian institution to answer this question. J Clin Endocrinol Metab 95:1516–1527
18. Tuttle RM, Vaisman F, Tronko MD (2011) Clinical presentation and clinical outcomesin Chernobyl- related paediatric thyroid cancers:what do we know now? What can we expect in the future? Clin Oncol 23:268–275
19. Giuffrida D, Gharib H (2000) Anaplastic thyroid carcinoma: current diagnosis and treatment. Ann Oncol 11:1083–1089
20. Cote GJ, Gagel RF (2003) Lessons learned from the management of a rare genetic cancer. N Engl J Med 349:1566–1568
21. Fusco A, Chiappetta G, Hui P, Garcia-Rostan G, Golden L et al (2002) Assessment of RET/PTC oncogene activation and clonality in thyroid nodules with incomplete morphological evidence of papillary carcinoma: a search for the early precursors of papillary thyroid cancer. Am J Pathol 160:2157–2167
22. García-Rostán G, Camp RL, Herrero A, Carcangiu ML, Rimm DL et al (2001) Beta-catenin dysregulation in thyroid neoplasms: down-regulation, aberrant nuclear expression, and

CTNNB1 exon 3 mutations are markers for aggressive tumor phenotypes and poor prognosis. Am J Pathol 158:987–996
23. Garcia-Rostan G, Zhao H, Camp RL, Pollan M, Herrero A et al (2003) ras mutations are associated with aggressive tumor phenotypes and poor prognosis in thyroid cancer. J Clin Oncol 21:3226–3235
24. Garcia-Rostan G, Costa AM, Pereira-Castro I, Salvatore G, Hernandez R et al (2005) Mutation of the PIK3CA gene in anaplastic thyroid cancer. Cancer Res 65:10199–10207
25. Tallini G, Garcia-Rostan G, Herrero A, Zelterman D, Viale G et al (1999) Downregulation of p27KIP1 and Ki67/Mib1 labeling index support the classification of thyroid carcinoma into prognostically relevant categories. Am J Surg Pathol 23:678–685
26. Santoro M, Melillo RM, Carlomagno F, Vechio G, Fusco A (2004) Minireview: RET: normal and abnormal functions. Endocrinology 145:5448–5451
27. Santoro M, Carlomagno F (2006) Drug insight: small-molecule inhibitors of protein kinases in the treatment of thyroid cancer. Nat Clin Pract Endocrinol Metab 2:42–52
28. Manié S, Santoro M, Fusco A, Billaud M (2001) The RET receptor: function in development and dysfunction in congenital malformation. Trends Genet 17:580–589
29. Ciampi R, Nikiforov YE (2007) RET/PTC rearrangements and BRAF mutations in thyroid tumorigenesis. Endocrinology 148:936–941
30. Fusco A, Grieco M, Santoro M, Berlingieri MT, Pilotti S et al (1987) A new oncogene in human thyroid papillary carcinomas and their lymph-nodal metastases. Nature 328:170–172
31. Grieco M, Santoro M, Berlingieri MT, Melillo RM, Donghi R et al (1990) PTC is a novel rearranged form of the ret proto-oncogene and is frequently detected in vivo in human thyroid papillary carcinoma. Cell 60:557–563
32. Santoro M, Carlomagno F, Hay ID, Herrmann MA, Grieco M et al (1992) Ret oncogene activation in human thyroid neoplasms is restricted to the papillary cancer subtype. J Clin Invest 89:1517–1522
33. Tallini G, Santoro M, Helie M, Carlomagno F, Salvatore G et al (1998) RET/PTC oncogene activation defines a subset of papillary thyroid carcinomas lacking eveidence of progression to poorly differentiated or undifferentiated tumor phenotypes. Clin Cancer Res 4:287–294
34. Nikiforov YE, Nikiforova MN (2011) Molecular genetics and diagnosis of thyroid cancer. Nat Rev Endocrinol 7:569–580
35. Nikiforova MN, Stringer JR, Blough R, Medvedovic M, Fagin JA et al (2000) Proximity of chromosomal loci that participate in radiation-induced rearrangements in human cells. Science 290:138–141
36. Santoro M, Dathan NA, Berlingieri MT, Borganzone I, Paulin C et al (1994) Molecular characterization of RET/PTC3; a novel rearranged version of the RET proto-oncogene in human thyroid papillary carcinoma. Oncogene 9:509–516
37. Borganzone I, Butti MG, Coronelli S, Borrello MG, Santoro M et al (1994) Frequent activation of ret protooncogene by fusion with a new activating gene in papillary thyroid carcinomas. Cancer Res 54:2979–2985
38. Santoro M, Melillo RM, Grieco M, Berlingieri MT, Vecchio G et al (1993) The TRK and RET tyrosine kinase oncogenes cooperate with ras in the neoplastic transformation of a rat thyroid epithelial cell line. Cell Growth Differ 4:77–84
39. De Vita G, Zannini M, Cirafici AM, Melillo RM, Di Lauro R et al (1998) Expression of RET/PTC1 oncogene impairs the activity of TTF-1 and Pax-8 thyroid transcription factors. Cell Growth Differ 9:97–103
40. Santoro M, Chiappetta G, Cerrato A, Salvatore D, Zhang L et al (1996) Development of thyroid papillary carcinomas secondary to tissue-specific expression of the RET/PTC1 oncogene in transgenic mice. Oncogene 12:1821–1826
41. Romei C, Elisei R (2012) RET/PTC translocations and clinico-pathological features in human papillary thyroid carcinoma. Front Endocrinol 3:54
42. Marotta V, Guerra A, Sapio MR, Vitale M (2011) RET/PTC rearrangement in benign and malignant thyroid disease: a clinical standpoint. Eur J Endocrinol 165:499–507

43. Ishizaka Y, Kobayashi S, Ushijima T, Hirohashi S, Sugimura T et al (1991) Detection of retTPC/PTC transcripts in thyroid adenomas and adenomatous goiter by an RT-PCR method. Oncogene 6:1667–1672
44. Wirtschafter A, Schmidt R, Rosen D, Kundu N, Santoro M et al (1997) Expression of the RET/PTC fusion gene as a marker for papillary carcinoma in Hashimoto's thyroiditis. Laryngoscope 107:95–100
45. Sheils OM, O'Eary JJ, Uhlmann V, Lattich K, Sweeney EC (2000) ret/PTC-1 activation in Hashimoto thyroiditis. Int J Surg Pathol 8:185–189
46. Elisei R, Romei C, Vorontsova T, Cosci B, Veremeychik V et al (2001) RET/PTC rearrangements in thyroid nodules: studies in irradiated and not irradiated, malignant and benign thyroid lesions in children and adults. J Clin Endocrinol Metab 86:3211–3216
47. Chiappetta G, Toti P, Cetta F, Giuliano A, Pentimalli F et al (2002) The RET/PTC oncogene is frequently activated in oncocytic thyroid tumors (Hurthle cell adenomas and carcinomas), but not in oncocytic hyperplastic lesions. J Clin Endocrinol Metab 87:364–369
48. Sapio MR, Guerra A, Marotta V, Campanile E, Formisano R et al (2011) High growth rate of benign thyroid nodules bearing RET/PTC rearrangements. J Clin Endocrinol Metab 96:E916–E919
49. Rhoden KJ, Unger K, Salvatore G, Yilmaz Y, Vovk V et al (2006) RET/papillary thyroid cancer rearrangement in nonneoplastic thyrocytes: follicular cells of Hashimoto's thyroiditis share low-level recombination events with a subset of papillary carcinoma. J Clin Endocrinol Metab 91:2414–2423
50. Nikiforov YE (2002) RET/PTC rearrangement in thyroid tumors. Endocr Pathol 13:3–16
51. Tallini G, Asa SL (2001) RET oncogene activation in papillary thyroid carcinoma. Adv Anat Pathol 8:345–354
52. Thomas GA, Bunnell H, Cook HA, Williams ED, Nerovnya A et al (1999) High prevalence of RET/PTC rearrangements in Ukrainian and Belarussian post-Chernobyl thyroid papillary carcinomas: a strong correlation between RET/PTC3 and the solid-follicular variant. J Clin Endocrinol Metab 84:4232–4238
53. Saad A, Falciglia M, Steward DL, Nikiforov YE (2004) Amiodarone-induced thyrotoxicosis and thyroid cancer:clinical, immunohistochemical and molecular genetic studies of a case and review of the literature. Arch Pathol Lab Med 128:807–810
54. Sugg SL, Ezzat S, Zheng L, Freeman JL, Rosen IB et al (1999) Oncogene profile of papillary thyroid carcinoma. Surgery 125:46–52
55. Mochizuki K, Kondo T, Nakazawea T, Iwashima M, Kawasaki T et al (2010) RET rearrangements and BRAF mutations in undifferentiated thyroid carcinomas having papillary carcinoma components. Histopathology 57:444–450
56. Celetti A, Cerrato A, Merolla F, Vitagliano D, Vecchio G et al (2004) H4(D10S170), a gene frequently rearranged with RET in papillary thyroid carcinomas: functional characterization. Oncogene 23:109–121
57. Merolla F, Pentimalli F, Pacelli R, Vecchio G, Fusco A et al (2007) Involvement of H4(D10S170) protein in ATM-dependent response to DNA damage. Oncogene 26:6167–6175
58. Kirschner LS, Carney JA, Pack SD, Taymans SE, Giatzakis C et al (2000) Mutations of the gene encoding the protein kinase A type I-a regulatory subunit in patients with the Carney complex. Nat Genet 26:89–92
59. Boikos SA, Stratakis CA (2006) Carney complex: pathology and molecular genetics. Neuroendocrinology 83:189–199
60. Kollara A, Kahn HJ, Marks A, Brown TJ (2001) Loss of androgen receptor associated protein 70 (ARA70) expression in a subset of HER2-positive breast cancers. Breast Cancer Res Treat 67:245–253
61. Li P, Yu X, Ge K, Melamed J, Roeder RG et al (2002) Heterogeneous expression and functions of androgen re- ceptor co-factors in primary prostate cancer. Am J Pathol 161:1467–1474

62. Ligr M, Li Y, Zou X, Daniels G, Melamed J et al (2010) Tumor suppressor function of androgen receptor coactivator ARA70a in prostate cancer. Am J Pathol 176:1891–1900
63. Klugbauer S, Rabes HM (1999) The transcription coactivator HTIF1 and a related protein are fused to the RET receptor tyrosine kinase in childhood papillary thyroid carcinomas. Oncogene 18:4388–4393
64. Wong N, Lai P, Lee SW, Fan S, Pang E et al (1999) Assessment of genetic changes in hepatocellular carcinoma by comparative genomic hybridization analysis: relationship to disease stage, tumor size, and cirrhosis. Am J Pathol 154:37–43
65. Khetchoumian K, Teletin M, Tisserand J, Mark M, Herquel B et al (2007) Loss of Trim24 (Tif1a) gene function confers oncogenic activity to retinoic acid receptor alpha. Nat Genet 39:1500–1506
66. Pierotti MA, Bongarzone I, Borrello MG, Mariani C, Miranda C et al (1995) Rearrangements of TRK proto-oncogene in papillary thyroid carcinomas. J Endocrinol Invest 18:130–133
67. Ciampi R, Knauf JA, Kerler R, Gandhi M, Zhu Z et al (2005) Oncogenic AKAP9-BRAF fusion is a novel mechanism of MAPK pathway activation in thyroid cancer. J Clin Invest 115:94–101
68. Gandhi M, Evdokimova V, Nikiforov YE (2010) Mechanisms of chromosomal rearrangements in solid tumors: the model of papillary thyroid carcinoma. Mol Cell Endocrinol 321:36–43
69. Kawamoto Y, Takeda K, Okuno Y, Yamakawa Y, Ito Y et al (2004) Identification of RET autophosphorylation sites by mass spectrometry. J Biol Chem 279:14213–14224
70. Iwashita T, Asai N, Murakami H, Matsuyama M, Takahashi M (1996) Identification of tyrosine residues that are essential for transforming activity of the ret proto-oncogene with MEN2A or MEN2B mutation. Oncogene 12:481–487
71. Knowles PP, Murray-Rust J, Kjaer S, Scott RP, Hanrahan S et al (2006) Structure and chemical inhibition of the RET tyrosine kinase domain. J Biol Chem 281:33577–33587
72. Pandey A, Liu X, Dixon JE, Di Fiore PP, Dixit VM (1996) Direct association between the Ret receptor tyrosine kinase and the Src homology 2-containing adapter protein Grb7. J Biol Chem 271:10607–10610
73. Donatello S, Fiorino A, Degl'innocenti D, Alberti L, Miranda C et al (2007) SH2B1beta adaptor is a key enhancer of RET tyrosine kinase signaling. Oncogene 26:6546–6559
74. Borrello MG, Alberti L, Arighi E, Bongarzone I, Battistini C et al (1996) The full oncogenic activity of Ret/ptc2 depends on tyrosine 539, a docking site for phospholipase Cgamma. Mol Cell Biol 16:2151–2163
75. Jain S, Encinas M, Johnson EM Jr, Milbrandt J (2006) Critical and distinct roles for key RET tyrosine docking sites in renal development. Genes Dev 20:321–333
76. Encinas M, Crowder RJ, Milbrandt J, Johnson EM Jr (2004) Tyrosine 981, a novel ret autophosphorylation site, binds c-Src to mediate neuronal survival. J Biol Chem 279:18262–18269
77. Segouffin-Cariou C, Billaud M (2000) Transforming ability of MEN2A-RET requires activation of the phosphatidylinositol 3-kinase/AKT signaling pathway. J Biol Chem 275:3568–3576
78. Melillo RM, Castellone MD, Guarino V, De Falco V, Cirafici AM et al (2005) The RET/PTC-RAS-BRAF linear signaling cascade mediates the motile and mitogenic phenotype of thyroid cancer cells. J Clin Invest 115:1068–1081
79. Castellone MD, Cirafici AM, De Vita G, De Falco V, Malorni L et al (2003) Ras-mediated apoptosis of PC CL 3 rat thyroid cells induced by RET/PTC oncogenes. Oncogene 22:246–255
80. Jijiwa M, Fukuda T, Kawai K, Nakamura A, Kurokawa K et al (2004) A targeting mutation of tyrosine 1062 in Ret causes a marked decrease of enteric neurons and renal hypoplasia. Mol Cell Biol 24:8026–8036

81. Wong A, Bogni S, Kotka P, de Graaff E, D'Agati V et al (2005) Phosphotyrosine 1062 is critical for the in vivo activity of the Ret9 receptor tyrosine kinase isoform. Mol Cell Biol 25:9661–9673
82. Ichihara M, Murakumo Y, Takahashi M (2004) RET and neuroendocrine tumors. Cancer Lett 204:197–211
83. Asai N, Murakami H, Iwashita T, Takahashi M (1996) A mutation at tyrosine 1062 in MEN2A-Ret and MEN2B-Ret impairs their transforming activity and association with shc adaptor proteins. J Biol Chem 271:17644–17649
84. Melillo RM, Santoro M, Ong SH, Billaud M, Fusco A, Hadari YR, Schlessinger J, Lax I (2001) Docking protein FRS2 links the protein tyrosine kinase RET and its oncogenic forms with the mitogen-activated protein kinase signaling cascade. Mol Cell Biol 21:4177–4187
85. De Falco V, Castellone MD, De Vita G, Cirafici AM, Hershman JM et al (2007) RET/papillary thyroid carcinoma oncogenic signaling through the Rap1 small GTPase. Cancer Res 67:381–390
86. Uchida M, Enomoto A, Fukuda T, Kurokawa K, Maeda K et al (2006) Dok-4 regulates GDNF-dependent neurite outgrowth through downstream activation of Rap1 and mitogen-activated protein kinase. J Cell Sci 119(Pt 15):3067–3077
87. Knauf JA, Kuroda H, Basu S, Fagin JA (2003) RET/PTC-induced dedifferentiation of thyroid cells is mediated through Y1062 signaling through SHC-RAS-MAP kinase. Oncogene 22:4406–4412
88. Mitsutake N, Knauf JA, Mitsutake S, Mesa C Jr, Zhang L et al (2005) Conditional BRAFV600E expression induces DNA synthesis, apoptosis, dedifferentiation, and chromosomal instability in thyroid PCCL3 cells. Cancer Res 65:2465–2473
89. Pelicci G, Troglio F, Bodini A, Melillo RM, Pettirossi V et al (2002) The neuron-specific Rai (ShcC) adaptor protein inhibits apoptosis by coupling Ret to the phosphatidylinositol 3-kinase/Akt signaling pathway. Mol Cell Biol 20:7351–7363
90. Miyagi E, Braga-Basaria M, Hardy E, Vasko V, Burman KD et al (2004) Chronic expression of RET/PTC3 enhances basal insulin-stimulated PI3 kinase/AKT signaling and increases IRS-2 expression in FRTL-5 thyroid cells. Mol Carcinog 41:98–107
91. Jung HS, Kim DW, Jo YS, Chung HK, Song JH et al (2005) Regulation of protein kinase B tyrosine phosphorylation by thyroid-specific oncogenic RET/PTC kinases. Mol Endocrinol 19:2748–2759
92. De Falco V, Guarino V, Malorni L, Cirafici AM, Troglio F et al (2005) RAI(ShcC/N-Shc)-dependent recruitment of GAB 1 to RET oncoproteins potentiates PI 3-K signalling in thyroid tumors. Oncogene 24:6303–6313
93. Castellone MD, De Falco V, Rao DM, Bellelli R, Muthu M et al (2009) The beta-catenin axis integrates multiple signals downstream from RET/papillary thyroid carcinoma leading to cell proliferation. Cancer Res 69:1867–1876
94. Brophy PD, Ostrom L, Lang KM, Dressler GR (2001) Regulation of ureteric bud outgrowth by Pax2-dependent activation of the glial derived neurotrophic factor gene. Development 128:4747–4756
95. Clarke JC, Patel SR, Raymond RM Jr, Andrew S, Robinson BG et al (2006) Regulation of c-Ret in the developing kidney is responsive to Pax2 gene dosage. Hum Mol Genet 15:3420–3428
96. Kume T, Deng K, Hogan BL (2000) Murine forkhead/winged helix genes Foxc1 (Mf1) and Foxc2(Mfh1) are required for the early organogenesis of the kidney and urinary tract. Development 127:1387–1395
97. Grieshammer U, Le M, Plump AS, Wang F, Tessier-Lavigne M et al (2004) SLIT2-mediated ROBO2 signaling restricts kidney induction to a single site. Dev Cell 6:709–717
98. Miyazaki Y, Oshima K, Fogo A, Hogan BL, Ichikawa I (2000) Bone morphogenetic protein 4 regulates the budding site and elongation of the mouse ureter. J Clin Invest 105:863–873
99. Basson MA, Akbulut S, Watson-Johnson J, Simon R, Carroll TJ et al (2005) Sprouty1 is a critical regulator of GDNF/RET-mediated kidney induction. Dev Cell 8:229–239

100. Ishida M, Ichihara M, Mii S, Jijiwa M, Asai N et al (2007) Sprouty2 regulates growth and differentiation of human neuroblastoma cells through RET tyrosine kinase. Cancer Sci 98:815–821
101. Kim D, Dressler GR (2007) PTEN modulates GDNF/RET mediated chemotaxis and branching morphogenesis in the developing kidney. Dev Biol 307:290–299
102. Hempelmann LH, Pifer JW, Burke GJ, Terry R, Ames WR (1967) Neoplasms in persons treated with X rays in infancy for thymic enlargement. A report of the third follow up survey. J Natl Cancer Inst 38:317–341
103. Bielshowsky F (1953) Chronic iodine deficiency as cause of neoplasia in thyroid and pituitary of aged rats. Br J Cancer 7:203–213
104. Duffy BJ Jr, Fitzgerald PJ (1950) Cancer of the thyroid in children: a report of 28 cases. J Clin Endocrinol Metab 10:1296–1308
105. Williams D (2008) Twenty years' experience with post-Chernobyl thyroid cancer. Best Pract Res Clin Endocrinol Metab 22:1061–1073
106. Wood JW, Tamagaki H, Neriishi S, Sato T, Sheldon WF et al (1969) Thyroid carcinoma in atomic bomb survivors Hiroshima and Nagasaki. Am J Epidemiol 89:4–14
107. Conard RA, Dobyns BM, Sutow WW (1970) Thyroid neoplasia as late effect of exposure to radioactive iodine in fallout. JAMA 214:316–324
108. Shore RE, Woodward E, Hildreth N, Dvoresky P, Hempelmann L et al (1985) Thyroid tumors following thymus irradiation. J Natl Cancer Inst 74:1177–1184
109. Williams ED, Abrosimov A, Bogdanova T, Demidchik EP, Ito M (2008) Morphologic characteristics of Chernobyl-related childhood papillary thyroid carcinomas are independent of radiation exposure but vary with iodine intake. Thyroid 18:847–852
110. Cancer incidence in five continents (1987), vol V. IARC Scientific Publications, pp 1–970
111. Baverstock K, Egloff B, Pinchera A, Ruchti C, Williams D (1992) Thyroid cancer after Chernobyl. Nature 359:21–22
112. Kazakov US, Demidchick EP, Astakhova LN (1992) Thyroid cancer after Chernobyl. Nature 369:21
113. Williams D (1993) Radioiodine-induced thyroid cancer. Histopathology 23:387–389
114. Ito T, Seyama T, Iwamoto KS, Hayashi T, Mizuno T et al (1993) In vitro irradiation is able to cause RET oncogene rearrangement. Cancer Res 53:2940–2943
115. Caudill CM, Zhu Z, Ciampi R, Stringer JR, Nikiforov YE (2005) Dose-dependent generation of RET/PTC in human thyroid cells after in vitro exposure to gammaradiation: a model of carcinogenic chromosomal rearrangement induced by ionizing radiation. J Clin Endocrinol Metab 90:2364–2369
116. Mizuno T, Kyoizumi S, Suzuki T, Iwamoto KS, Seyama T (1997) Continued expression of a tissue specific activated oncogene in the early steps of radiationinduced human thyroid carcinogenesis. Oncogene 15:1455–1460
117. Mizuno T, Iwamoto KS, Kyoizumi S, Nagamura H, Shinohara T et al (2000) Preferential induction of RET/PTC1 rearrangement by X-ray irradiation. Oncogene 19:438–443
118. Gandhi M, Dillon LW, Pramanik S, Nikiforov YE, Wang YH (2010) DNA breaks at fragile sites generate oncogenic RET/PTC rearrangements in human thyroid cells. Oncogene 29:2272–2280
119. Roccato E, Bressan P, Sabatella G, Rumio C, Vizzotto L et al (2005) Proximity of TPR and NTRK1 rearranging loci in human thyrocytes. Cancer Res 65:2572–2576
120. El Hassani RA, Boufraquech M, Lagente Chevalier O, Weyemi U, Talbot M et al (2010) Role of H2O2 in RET/PTC1 chromosomal rearrangement produced by ionizing radiation in human thyroid cells. Cancer Res 70:4123–4132
121. Yang T, Namba H, Hara T, Takmura N, Nagayama Y et al (1997) p53 induced by ionizing radiation mediates DNA end-jointing activity, but not apoptosis of thyroid cells. Oncogene 14:1511–1519
122. Volpato CB, Martínez-Alfaro M, Corvi R, Gabus C, Sauvaigo S et al (2008) Enhanced sensitivity of the RET proto-oncogene to ionizing radiation in vitro. Cancer Res 68:8986–8992

123. Ito T, Seyama T, Iwamoto KS, Mizuno T, Tronko ND et al (1994) Activated RET oncogene in thyroid cancers of children from areas contaminated by Chernobyl accident. Lancet 344:259
124. Fugazzola L, Pilotti S, Pinchera A, Vorontsova TV, Mondellini P et al (1995) Oncogenic rearrangements of the RET proto-oncogene in papillary thyroid carcinomas from children exposed to the Chernobyl nuclear accident. Cancer Res 55:5617–5620
125. Klugbauer S, Lengfelder E, Demidchick EP, Rabes HM (1995) High prevalence of RET rearrangement in thyroid tumors of children from Belarus after the Chernobyl reactor accident. Oncogene 11:2459–2467
126. Santoro M, Thomas GA, Vecchio G, Williams GH, Fusco A et al (2000) Gene rearrangement and Chernobyl related thyroid cancers. Br J Cancer 82:315–322
127. Nikiforov YE, Rowland JM, Bove KE, Monforte-Munoz H, Fagin JA (1997) Distinct pattern of ret oncogene rearrangements in morphological variants of radiation-induced and sporadic thyroid papillary carcinomas in children. Cancer Res 57:1690–1694
128. Leeman Neill RJ, Brenner AV, Little MP, Bogdanova T, Hatch M et al (2013) RET/PTC and PAX8/PPRgamma chromosomal rearrangements in post-Chernobyl thyroid cancer and their association with iodine 131 radiation dose and other characteristics. Cancer 119:1792–1799
129. Ricarte-Filho JC, Li S, Garcia-rendueles MER, Montero Conte C, Voza F et al (2013) Identification of kinase fusion oncogenes in post-Chernobyl radiation-induced thyroid cancers. J Clin Invest 123:4935–4944
130. Bounacer A, Wicker R, Caillou B, Cailleux AF, Sarasin A et al (1997) High prevalence of activating ret proto-oncogene rearrangements in thyroid tumors from patients who had received external radiation. Oncogene 15:1263–1273
131. Alipov G, Ito M, Prouglo Y, Takamura N, Yamashita S (1999) RET proto-oncogene rearrangement in thyroid cancer around Semipalatinsk nuclear testing site. Nature 354:1529–1530
132. Hamatani K, Eguchi H, Ito R, Mukai M, Takahashi K et al (2008) RET/PTC rearrangements preferentially occurred in papillary thyroid cancer among atomic bomb survivors exposed to high radiation dose. Cancer Res 68:7176–7182
133. Ballerini P, Struski S, Cresson C, Prade N, Toujani S et al (2012) RET fusion genes are associated with chronic myelomonocytic leukemia and enhance monocytic differentiation. Leukemia 26:2384–2389
134. Bossi D, Carlomagno F, Pallavicini I, Pruneri G, Trubia M et al (2014) Functional characterization of a novel FGFR1OP-RET rearrangement in hematopoietic malignancies. Mol Oncol 8:221–231
135. Camos M, Esteve J, Jares P, Colomer D, Rozman M et al (2006) Gene expression profiling of acute myeloid leukemia with translocation t(8;16)(p11;p13) and MYST3-CREBBP rearrangement reveals a distinctive signature with a specific pattern of HOX gene expression. Cancer Res 66:6947–6954
136. Gattei V, Celetti A, Cerrato A, Degan M, De Iuliis A et al (1997) Expression of the RET receptor tyrosine kinase and GDNFR-alpha in normal and leukemic human hematopoietic cells and stromal cells of the bone marrow microenvironment. Blood 89:2925–2937
137. Ju YS, Lee WC, Shin JY, Lee S, Bleazard T et al (2012) A transforming KIF5B and RET gene fusion in lung adenocarcinoma revealed from whole-genome and transcriptome sequencing. Genome Res 22:436–445
138. Kohno T, Ichikawa H, Totoki Y, Yasuda K, Hiramoto M et al (2012) KIF5B-RET fusions in lung adenocarcinoma. Nat Med 18:375–377
139. Li F, Feng Y, Fang R, Fang Z, Xia J et al (2012) Identification of RET gene fusion by exon array analyses in "pan-negative" lung cancer from never smokers. Cell Res 22:928–931
140. Wang R, Hu H, Pan Y, Li Y, Ye T et al (2012) RET fusions define a unique molecular and clinicopathologic subtype of non-small-cell lung cancer. J Clin Oncol 30:4352–4359

141. Unger K, Wienberg J, Riches A, Hieber L, Walch A, Brown A, O'Brien PCM, Briscoe P, Gray L, Rodriguez E, Jackl G, Knijnenburg J, Tallini G, Ferguson-Smith M, Zitzelsberger H (2010) Novel gene rearrangements in transformed breast cells identified by high- resolution breakpoint analysis of chromosomal aberrations. Endocr Relat Cancer 17:87–98
142. Esseghir S, Todd SK, Hunt T, Poulsom R, Plaza-Menacho I et al (2007) A role for glial cell derived neurotrophic factor induced expression by inflammatory cytokines and RET/GFR-a 1 receptor up-regulation in breast cancer. Cancer Res 67:11732–11741
143. Spanheimer PM, Park JM, Askeland RW, Kulak MV, Woodfield GW et al (2014) Inhibition of RET increases the efficacy of antiestrogen and is a novel treatment strategy for luminal breast cancer. Clin Cancer Res 20:2115–2125
144. Gattelli A, Nalvarte I, Boulay A, Roloff TC, Schreiber M et al (2013) Ret inhibition decreases growth and metastatic potential of estrogen receptor positive breast cancer cells. EMBO Mol Med 5:1335–1350
145. Flavin R, Jackl G, Finn S, Smyth P, Ring M et al (2009) RET/PTC rearrangement occurring in primary peritoneal carcinoma. Int J Surg Pathol 17:187–197
146. Wiesner T, He J, Yelensky R, Esteve-Puig R, Botton T et al (2014) Kinase fusions are frequent in Spitz tumours and spitzoid melanomas. Nat Commun 5:3116
147. Rabes HM, Demidchik EP, Sidorow JD, Lengfelder E, Beimfohr C et al (2000) Pattern of radiation-induced RET and NTRK1 rearrangements in 191 post-chernobyl papillary thyroid carcinomas: biological, phenotypic, and clinical implications. Clin Cancer Res 6:1093–1103
148. Drilon A, Wang L, Hasanovic A, Suehara Y, Lipson D, Stephens P, Ross J, Miller V, Ginsberg M, Zakowski MF, Kris MG, Ladanyi M, Rizvi N (2013) Response to Cabozantinib in patients with RET fusion-positive lung adenocarcinomas. Cancer Discov 3:630–635
149. Shawver LK, Slamon D, Ullrich A (2002) Smart drugs: tyrosine kinase inhibitors in cancer therapy. Cancer Cell 1:117–123
150. Reichert JM, Valge-Archer VE (2007) Development trends for monoclonal antibody cancer therapeutics. Nat Rev Drug Discov 6:349–356
151. Salvatore G, Nagata S, Billaud M, Santoro M, Vecchio G et al (2002) Generation and characterization of novel monoclonal antibodies to the Ret receptor tyrosine kinase. Biochem Biophys Res Commun 294:813–817
152. Schlumberger M, Sherman SI (2009) Clinical trials for progressive differentiated thyroid cancer: patient selection, study design, and recent advances. Thyroid 19:1393–1400
153. Sherman SI (2009) Tyrosine kinase inhibitors and the thyroid. Best Pract Res Clin Endocrinol Metab 23:713–722
154. Wells SA, Robinson BC, Gagel RF, Dralle H, Fagin JA et al (2012) Vandetanib in patients with locally advanced or metastatic medullary thyroid cancer: a randomized, double-blind phase III trial. J Clin Oncol 30:134–141
155. Brilli L, Pacini F (2011) Targated therapy in refractory thyroid cancer: current achievements and limitations. Future Oncol 7:657–668

Chapter 17
Gene Fusions in Prostate Cancer

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Contents

17.1	Discovery of ETS Gene Fusions in Prostate Cancer	382
17.2	Diversity of ETS Gene Fusions in Prostate Cancer	383
17.3	Prevalence of ETS Gene Fusions in Prostate Cancer	384
17.4	Functional Characterization of ETS Gene Family Rearrangements	385
17.5	MAPK Pathway Gene Fusions in Prostate Cancer	386
17.6	Additional Gene Rearrangements in Prostate Cancer	387
17.7	Clinical Utility of Gene Rearrangement Status in Prostate Cancer	388
17.8	Gene Fusions as a Basis for Molecular Subtyping and Targeted Therapy in Prostate Cancer	391
17.9	Summary and Future Directions	394
17.10	Competing Interests	394
Acknowledgements		394
References		394

Abstract Recurrent chromosomal rearrangements resulting in the fusion of androgen regulated genes with ETS transcription factor family members *ERG* or *ETV1* are the most common molecular abnormality in prostate cancer. In the ensuing decade since this discovery, enormous progress has been made in understanding the diversity, biogenesis, and function of ETS gene fusions, both in vitro and in vivo. ETS gene fusions and associated co-occurring or mutually exclusive genetic events provide a rational basis for the comprehensive molecular subtyping of prostate cancer with potential utility for precision medicine approaches. As the most specific known biomarker in prostate cancer, ETS gene fusions may have utility in tissue based diagnosis, risk stratification of precursor lesions, early detection, and distin-

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guishing between clinically aggressive and indolent cases of prostate cancer. While the vast majority of recurrent gene rearrangements in prostate cancer involve the ETS gene family, additional low frequency fusion events involving known oncogenes *BRAF*, *KRAS*, *RAF1*, and *FGFR2* have also been described. These rare events suggest that small subsets of prostate cancer patients harbour fusion events with immediate clinical significance and are candidates for targeted therapy.

Keywords TMPRSS2:ERG • ETS genes • FGFR3 • RAS/RAF family • Early detection

17.1 Discovery of ETS Gene Fusions in Prostate Cancer

While the use of DNA microarrays for global gene expression profiling to characterize cancer transcriptomes successfully allowed for molecular classification, biomarker discovery, and prognostic categorization, this methodology identified relatively few causal cancer genes [1–4]. The development of a novel technique of DNA microarray interpretation, termed cancer outlier profile analysis (COPA) (Reviewed in [5, 6]), nominated two members of the ETS transcription factor family, *ERG* and *ETV1*, as high ranking expression outliers in multiple prostate cancer profiling studies [7]. In all of the prostate cancer samples investigated, overexpression of *ERG* and *ETV1* was mutually exclusive (i.e. a single tumour sample may overexpress either *ERG* or *ETV1*, but never both), suggesting functional redundancy.

Given that ETS transcription factors are driving gene fusion partners in Ewing's sarcoma (most commonly *FLI1*), the possibility that the high-level overexpression of *ERG* and *ETV1* in prostate cancer might similarly result from gene rearrangement was investigated. Exon-walking quantitative PCR demonstrated loss of expression of the 5′ exons of the respective ETS family member in both *ERG* and *ETV1* overexpressing prostate cancers, with subsequent 5′-RNA ligase-mediated rapid amplification of cDNA ends (5′-RACE) unexpectedly identifying replacement of these lost 5′ ends of the ETS gene with sequences from the 5′ untranslated region of *TMPRSS2* [7].

Given that *TMPRSS2* is a strongly androgen regulated gene, this discovery provided a mechanism for the marked ETS gene over-expression seen in prostate cancer, where the 5′ promoter elements of *TMPRSS2* are subverted to drive aberrant androgen mediated expression of *ERG* or *ETV1*. These gene fusions encode a nearly full length ETS transcription factor [6, 8] with the 5′ partner rarely encoding translated sequence (akin to *IGH* mediated over-expression of *MYC* in Burkitt's lymphoma), as shown in Fig. 17.1.

This initial analysis identified *ERG* or *ETV1* rearrangements in approximately half of all PSA-detected prostate cancers, making ETS family fusions the most prevalent gene fusion in human cancer.

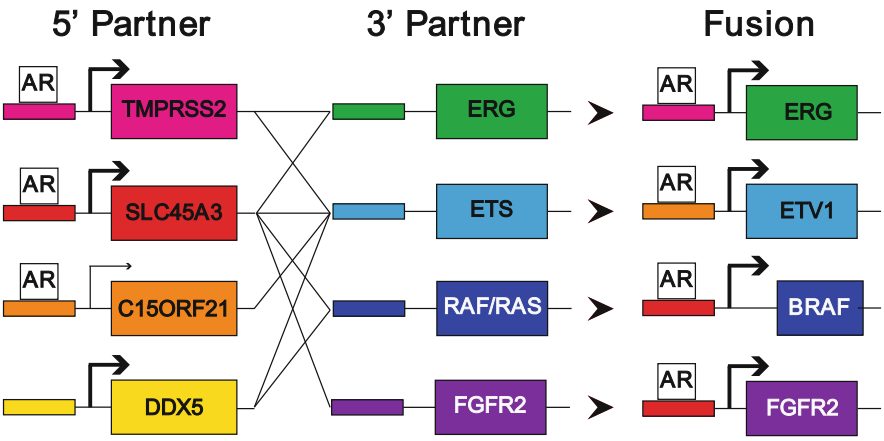

Fig. 17.1 Diversity of recurrent gene fusions identified in prostate cancer. Recurrent chromosomal rearrangements leading to gene fusions have been identified in >50 % of PSA-screened Caucasian cohorts. The most common 5′ fusion partner is the untranslated region of the androgen induced gene *TMPRSS2*. Additional recurrent 5′ fusion partners include other androgen induced genes (such as *SLC45A3*), an androgen repressed gene (*HMGN2P46*/*C15ORF21*) and ubiquitously expressed genes (such as *DDX5*). The most common 3′ fusion partner is a nearly full length ERG gene, a member of the ETS transcription factor family. Additional recurrent 3′ fusion partners include other ETS genes (such as *ETV1*), truncated or full length RAS/RAF family members (such as *BRAF*) and full length *FGFR2*

17.2 Diversity of ETS Gene Fusions in Prostate Cancer

In close to 90 % of prostate cancers harbouring ETS gene fusions, the 3′ fusion partner is *ERG*; with other *ETS* transcription factors participating at a lower frequency including (in decreasing prevalence) *ETV1* [7, 9], *ETV4* [10], *ETV5* [11], *ELK4* [12], and *FLI1* [13] (Fig. 17.1). Interestingly, the ETS family genes involved in fusions contain a highly conserved ETS DNA binding domain that is slightly divergent from the ETS family members that have not been observed in prostate cancer. In contrast to the 3′ partners from ETS gene family, numerous 5′ partners have been identified including known androgen driven genes (including *TMPRSS2*, *SLC45A3*, *KLK2*, *HERV-K_22q11.23* and *CANT1*), as well as one with an androgen-insensitive promoter (*DDX5*), one with a constitutively active promoter (*HNRPA2B1*), and a single case of an androgen-repressed promoter (*HMGN2P46*/*C15orf21*) [7, 10, 11, 14–18] (Fig. 17.1). Unlike other ETS gene members, the *SLC45A3*:*ELK4* fusion represents a read-through event of adjacent genes with no genomic disruption generating an oncogenic transcriptional chimaera [12, 19–21]. The roster of 5′ partners is still incomplete, and with the emergence of sensitive RNA-seq technology the identification of rare, novel 5′ partners will continue [19, 22, 23].

Despite the broad diversity of ETS family gene rearrangements in prostate cancer, *TMPRSS2*:*ERG* is by far the most prevalent fusion observed, with the large majority involving exon 1 of *TMPRSS2* (NM_005656) fused to exon 4 of *ERG* (NM_004449). The Exon 1 *TMPRSS2*: Exon 4 *ERG* gene fusion encodes an ERG protein product lacking only 4 N-terminal amino acids compared to wild type and, like full-length TMPRSS2 [14, 24, 25], expression of the fusion gene is androgen driven [7]. The androgen dependent nature of *TMPRSS2*:*ERG* gene expression is reflected in the massive overexpression of the ERG protein reported by immunohistochemistry (IHC) in prostate cancer tissue, with similar levels irrespective of the 5′ fusion partner, including non-*TMPRSS2* 5′ partners like *SLC45A3* [26–29].

Additional reported *TMPRSS2*:*ERG* splice variants include exons 2, 4, or 5 of *TMPRSS2* fused to exons 2, 3, or 5 of *ERG*, as well as novel *TMPRSS2* start sequences, and fusions with deletions of various internal ERG exons [7, 30–39]. As is the case with specific 5′ fusion partners, the high level of ERG protein expression also appears independent of individual *TMPRSS2*:*ERG* fusion splice variants [27]. Multiple reports have described associations between specific *TMPRSS2*:*ERG* splice variants and various biological phenotypes using model systems; however, given that multiple splice isoforms are frequently expressed in a single patient's tumour, the relevance of these findings to human prostate cancer is unclear [32, 38, 40–42].

17.3 Prevalence of ETS Gene Fusions in Prostate Cancer

In the initial report of ETS gene fusions from a PSA-screened radical prostatectomy cohort, *ERG* or *ETV1* rearrangements were reported in 79 % of cancers using fluorescence in situ hybridization (FISH) [7]. Subsequent studies have used a variety of techniques to assess the prevalence of ETS gene fusions in prostate cancer, including reverse transcription-PCR (RT), FISH, quantitative PCR (qPCR), and more recently, IHC. A recent summary of ETS family rearrangements looking across 10,779 cases from multiple studies report a prevalence of 47 % [43]. The variability in the reported prevalence of ETS rearrangements between studies underscores the importance of several factors for assessing for ETS gene fusions, including the method of detection, source of specimen, and cohort characteristics (including race/ethnicity, population vs PSA screened, etc.) and has been extensively reviewed [6]. The method of detection utilised in a study can dramatically influence the 5′ partners, 3′ ETS genes, and specific gene fusions detected. For example, RT-PCR based methods for fusion detection have limited sensitivity for detecting novel 5′ partners and can under-report rearrangement prevalence, as was the case with *ETV1* [9, 30, 33, 37, 44]. Similarly, the development of ERG antibodies for IHC as a robust marker of *ERG* gene fusion has facilitated the screening of larger cohorts [27, 45], but this approach precludes detection of rearrangements of other ETS family members.

Additionally, the method used to obtain tissue can influence ETS gene fusion detection, particularly *ERG* rearrangements. Tissue acquired via transurethral resection of the prostate (TURP) procedures is primarily from the central portion of the gland (transition zone). Multiple studies have demonstrated that transition zone tumours consistently have lower rates of *ERG* fusions, from 12 to 18 % [46–52]. This suggests that alternative genetic mechanisms drive these tumours compared to those found in the outer portion of the gland (peripheral zone), which are typically sampled by core needle biopsy after PSA screening and show *ERG* rearrangement rates of 40 % or higher [29, 53–55]. These differences also confound comparison of PSA-screened and population based studies, which are typically composed of biopsy and TURP specimens, respectively.

Finally, the construction of the cohort studied can profoundly impact the rate of ETS rearrangement detections. Non-Caucasian patients reliably demonstrate lower rates of *ERG* fusions across numerous studies [53, 56–63], suggesting that further study of diverse demographic groups will likely uncover additional molecular subtypes of prostate cancer. Recent reports also demonstrate that ETS fusions are more frequent in younger patients and those with lower PSA [64, 65].

17.4 Functional Characterization of ETS Gene Family Rearrangements

ETS gene expression promotes malignant phenotypes and is critical for prostate cancer cell survival. In vitro studies using the VCaP cell line, (which naturally harbours the *TMPRSS2*:*ERG* rearrangement [66]), show *TMPRSS2*:*ERG* directed siRNA inhibits transcription of ETS dependent genes, cell growth, invasion, metastasis and xenograft tumour growth [42, 67]. Forced overexpression of truncated *ERG*, *ETV1*, *ETV4* or *ETV5* in benign immortalised prostate epithelial cells drives a unique ETS transcriptional programme promoting cell invasion [7, 10, 11, 68, 69].

In vivo work demonstrates a functional role for ETS gene fusions in prostate cancer pathogenesis, consistent with its evolutionary selection. Multiple studies utilising mice with *ERG* or *ETV1* under androgen regulation exhibit neoplastic precursor lesions (similar to prostatic intraepithelial neoplasia (PIN) in humans) with variable penetrance, but do not develop invasive carcinoma [5, 9, 70–76]. Combining *ERG* or *ETV1* overexpression with deletion of the *PTEN* tumour suppressor results in accelerated prostate carcinogenesis in some mice backgrounds [71, 72, 77]. Similarly, mouse prostate epithelial cells engineered to overexpress *ERG* and the androgen receptor (*AR*) form invasive cancer when transplanted into donor mice [74]. Taken together, these findings suggest ETS gene fusions accelerate prostate carcinogenesis; however, these model systems do not progress beyond local disease preventing investigation of the contribution of ETS genes to metastasis and advanced disease. Critically, in human prostate cancer tissues, ETS gene fusions are an early event, likely mediating the transition from high grade prostatic intraepithelial

neoplasia (HGPIN) to invasive cancer, and are not observed more frequently in advanced compared to localised prostate cancer [6, 78–80].

17.5 MAPK Pathway Gene Fusions in Prostate Cancer

Signalling via the MAPK pathway is critical for human carcinogenesis with activation of individual pathway members identified in a wide variety of cancer types. In prostate cancer specifically, overexpression of RAS in LNCaP prostate cancer cell lines promoted an androgen hypersensitive state in vitro and significantly advanced tumour growth and progression in xenograft models [81]. Conversely, abrogating RAS signalling restores androgen sensitivity to hormone refractory prostate cancer cell lines [82]. Additionally, large scale integrative genomic profiling has shown that distant metastases of prostate cancer demonstrate significant upregulation of MAPK member expression at high frequencies, although mutations at recurrent hotspots in *KRAS* and *BRAF* are relatively uncommon [83–85].

In 2010, using paired-end whole transcriptome sequencing, gene fusions involving *BRAF* and *RAF1* were identified in two ETS rearrangement negative prostate cancer samples (Fig. 17.1) [23]. One case involved an intrachromosomal rearrangement resulting in the fusion of the untranslated exon 1 of *SLC45A3* as the 5′ partner with exon 8 of *BRAF* as the 3′ partner. As noted above, *SLC45A3* is an androgen dependent gene known to act as a 5′ fusion partner with multiple ETS family members including *ERG*, *ETV1*, *ETV5*, and *ELK4* [9, 11, 12, 19, 86, 87]. This *BRAF* fusion is therefore under androgen dependent control expressing the presumed constitutively active C-terminal kinase domain of BRAF without the N-terminal Ras binding regulatory domain. Expression of the *SLC45A3-BRAF* fusion gene in RWPE cells (human benign immortalised prostate epithelial cells) generates a 37 kDa protein, resulting in increased proliferation, invasion, and xenograft tumour formation, emphasizing the oncogenic potential of this fusion [23].

The second case identified a balanced reciprocal translocation resulting involving *ESRP1*, a regulator of mRNA splicing with *RAF1* (or *CRAF*) (Fig. 17.1). The *ESRP1-RAF1* fusion brings together exon 13 of *ESRP1* (including its RNA recognition motifs) with exon 6 of *RAF1* (including the C-terminal kinase domain of *RAF1*). The lack of the N-terminal RAS binding domain again suggests constitutive activation of RAF1 kinase activity in the fusion protein, but the functional significance of the RNA binding domains from *ESRP1* is unclear [23]. Interestingly, *ESRP1* is not an androgen regulated gene unlike the majority of 5′ fusion partners noted in prostate cancer. Furthermore, the reciprocal gene arrangement of *RAF1-ESRP1* was also detected in this patient. Similar to the *SLC45A3-BRAF* fusion, expression of *ESRP1-RAF1* in RWPE cells generates a 120 kDa fusion protein that promotes proliferation and cell invasion in vitro. Additionally, expression of both fusion proteins resulted in increased activating phosphorylation of downstream signalling proteins such as MEK and ERK.

Using break-apart FISH for *BRAF* and *RAF1*, 6 of 349 prostate cancer cases showed *BRAF* alterations (5 rearrangements and 1 deletion of the 5′ probe) and 4 of 450 prostate cancer cases showed *RAF1* disruption (including 1 rearrangement and 3 deletions of the 3′ probe), suggesting a prevalence of 1–2 % [23]. Interestingly, these additionally discovered cases did not involve *SLC45A3* or *ESRP1*, signifying that, similar to ETS family rearrangements, this class of gene fusions has a heterogeneous group of 5′ partners. This was supported by Beltran et al. who subsequently reported an *EPB41*:*BRAF* fusion identified through targeted sequencing in clinically localised prostate cancer with Paneth cell–like differentiation [88]. Likewise, *BRAF* and *RAF1* fusions were detected by FISH at 2.5 % and 1.5 %, respectively, in a cohort of 218 Chinese men with prostate cancer [89]. Of note, the vast majority of cases with *BRAF* or *RAF1* rearrangements were associated with aggressive clinical behaviour and high tumour grade [23, 88, 89].

In addition to the low frequency but recurrent rearrangements involving *BRAF* and *RAF1*, one other very low frequency fusion involving MAPK pathway members has been described involving *KRAS* (Fig. 17.1). Using the knowledge that sites of genetic rearrangement are often secondarily amplified, Wang et al. used an array comparative genomic hybridization (aCGH) dataset of ten prostate cancer cell lines to identify amplifications near putative breakpoints to nominate novel fusion proteins [90]. Within the prostate cancer cell line DU145, a fusion between exon 3 of *UBE2L3* (which encodes for an ubiquitin conjugating enzyme) at the 5′ end and exon 2 of *KRAS* at the 3′ side was confirmed. Overexpression of the *UBE2L3-KRAS* fusion induced oncogenic phenotypes in RWPE cells in a MEK/ERK independent fashion [90]. Using both break apart FISH and aCGH breakpoint analysis, *KRAS* rearrangements were identified in 2 of 62 metastatic human prostate cancer samples; however, no *KRAS* aberrations were identified in 259 clinically localised primary tumours.

The fusions involving MAPK pathway members are essentially exclusive with ETS gene family rearrangements. A possible biologic explanation of this phenomenon comes from reports demonstrating ETS transcription factors, such as ETV1, are activated downstream of RAF/MEK/ERK signalling [91–94], and the findings that ETS fusions mimic MAPK signalling [69], providing a redundant pathway in prostate cancer cells. Critically, the discovery of recurrent gene rearrangements involving MAPK signalling molecules have generated considerable interest despite their low frequency due to the availability of existing FDA approved therapies that target these oncogenes (discussed below).

17.6 Additional Gene Rearrangements in Prostate Cancer

The proliferation of RNA-seq technology has revealed additional, non-ETS, non-MAPK pathway gene rearrangements in prostate cancer involving a number of loci. Whole transcriptome profiling of Chinese men with prostate cancer (known to have a lower prevalence of ETS gene rearrangements compared to Caucasians, discussed

above) identified multiple novel fusion events involving *USP9Y-TTY15*, *CTAGE5-KHDRBS3*, *RAD50-PDLIM4*, and *SDK1-AMACR* [95]. The *USP9Y-TTY15* fusion transcript represents a transcriptional read-through event between adjacent genes and produces no predicted open reading frames, leading to speculation that it functions as a long non-coding RNA, a class of molecules that have drawn recent attention in prostate cancer [96–98]. The high frequency of the *USP9Y-TTTY15*, *CTAGE5-KHDRBS3*, *RAD50-PDLIM4*, and *SDK1-AMACR* fusions in this study of Chinese men underscores the decreased frequency of ETS fusions in non-European men (which was present in 18.5 % of study patients) and suggests these novel rearrangements may have a role in prostate cancer pathogenesis in this population. Importantly, however, these fusions have yet to be functionally characterized or validated at the genomic level, and numerous chimaeric RNAs have been identified in prostate cancer without functional characterization [99].

The recent discovery of a *SLC45A3-FGFR2* interchromosomal gene fusion in a metastatic prostate cancer patient arose from an integrative, comprehensive clinical sequencing project named MI-ONCOSEQ [100, 101]. *SLC45A3*, as discussed above, is an androgen responsive gene and frequently acts as a 5′ fusion partner for *ETS* and *BRAF* fusions in prostate cancer. Oncogenic point mutations and amplifications of *FGFR2* have been associated with breast, gastric, colon, endometrial, and ovarian cancers [102]. Similar to ETS family rearrangements, the *SLC45A3-FGFR2* fusion is predicted to drive expression of full-length FGFR2 from the untranslated exon 1 of *SLC45A3* in an androgen dependent manner (Fig. 17.1). Supporting this finding was the outlier expression of *FGFR2* noted in this case compared to a cohort of 84 other prostate cancer specimens [101]. Outlier expression of *FGFR2* has been noted rarely ($\ll$1 %) in other prostate cancer cohorts [103], hinting that this fusion event may be present at low frequency outside this index patient.

17.7 Clinical Utility of Gene Rearrangement Status in Prostate Cancer

Given the extraordinary specificity of ETS gene rearrangements for prostate cancer, they have been extensively evaluated as possible biomarkers for use in the clinical setting as well as a possible therapeutic target. However, due to the fact that gene fusions appear in ~50 % of prostate cancer foci, there are obvious limits to their sensitivity as a biomarker. It is also important to consider that prostate cancer is typically multifocal, and the presence of a gene fusion can be variable across tumour foci, as they are genetically distinct clones. Despite these caveats, many studies have investigated the application of gene rearrangement status in use for early disease detection, diagnostic adjuncts, and risk stratification.

In contrast to PSA, the products of ETS gene fusions are not secreted and, therefore, not detectable in serum. However, *TMPRSS2*:*ERG* transcripts can be identified in the urine of men with prostate cancer following digital rectal exam [104–115].

The detection of such a highly cancer specific in a noninvasive setting makes *TMPRSS2*:*ERG* detection in the urine an appealing target for use in screening/early detection of prostate cancer.

The development and validation of a clinical grade, transcription mediated amplification (TMA) based assay for *TMPRSS2*:*ERG* in whole urine following digital rectal exam found that higher levels of urine *TMPRSS2*:*ERG* were associated with negative prognostic indicators for prostate cancer including increased tumour size, high Gleason score at prostatectomy, and upgrading of biopsy Gleason grade at prostatectomy [112]. This test yields a *TMPRSS2*:*ERG* continuous score (urine *TMPRSS2*:*ERG* transcript number/urine *PSA* transcript number) for dynamic and quantifiable detection of *TMPRSS2*:*ERG* transcripts, similar to the clinically available Progensa PCA3 test [116]. Combining the TMA based *TMPRSS2*:*ERG* assay with urine *PCA3* improved the ability of the Prostate Cancer Prevention Trial risk calculator (PCPTrc) to identify men with prostate cancer who were biopsied following urine testing [112]. Similarly, the urine *TMPRSS2*:*ERG* level, urine *PCA3* level, and PSA density were all correlated with prostate cancer on follow up biopsy presence of cancer on biopsy in a multivariate model, with nearly equivalent areas under the curve (AUC) for TMPRSS2:ERG (0.67) and *PCA3* (0.66) [114]. Lin et al. likewise found that both urine T2:ERG and PCA3 may have utility in risk prediction for men on active surveillance [117]. Young et al. compared the urine *TMPRSS2*:*ERG* levels with the tissue expression of ERG in matched prostatectomy specimens via immunohistochemistry, showing that urine score was strongly correlated to the total volume of tumour expressing ERG and to the number of foci present expressing ERG [118]. Taken together, these data support the cancer specificity of urine TMPRSS2:ERG, and when combined with *PCA3*, provides improved ability to identify men with prostate cancer compared to existing prediction models.

Diagnosis of prostate cancer is typically made by a surgical pathologist reviewing core biopsies using cytological and morphological features of glandular cells to determine the presence of carcinoma. In certain situations, the morphologic evidence for carcinoma is qualitatively or quantitatively insufficient, and immunohistochemistry is used as a diagnostic adjunct. Most commonly, antibodies to AMACR, an androgen responsive protein overexpressed in most prostate cancers [3], and markers of basal cells (such as p63 and high molecular weight cytokeratin), which are typically lost in cancer foci, are used. Although useful for aiding in prostate cancer diagnosis, these markers can be aberrantly expressed in benign glands, carcinoma mimickers, and can be absent in carcinoma (in up to 20 % of cases) [119–122]. Many studies have investigated ERG immunohistochemistry as a diagnostic aid and have demonstrated positive ERG staining to be at least 99.99 % specific for prostate carcinoma, with exceptionally rare staining in benign glands, which are nearly always in close proximity to ERG positive carcinoma (Fig. 17.2) [26–29, 45, 47, 54, 55, 123–126].

High grade prostatic intraepithelial neoplasia (HGPIN), an entity where cytologically atypical cells line architecturally normal glands, is the presumed precursor to prostate cancer and can be difficult to distinguish from invasive carcinoma in the biopsy setting [127]. While almost all cases of HGPIN express AMACR [120, 128];

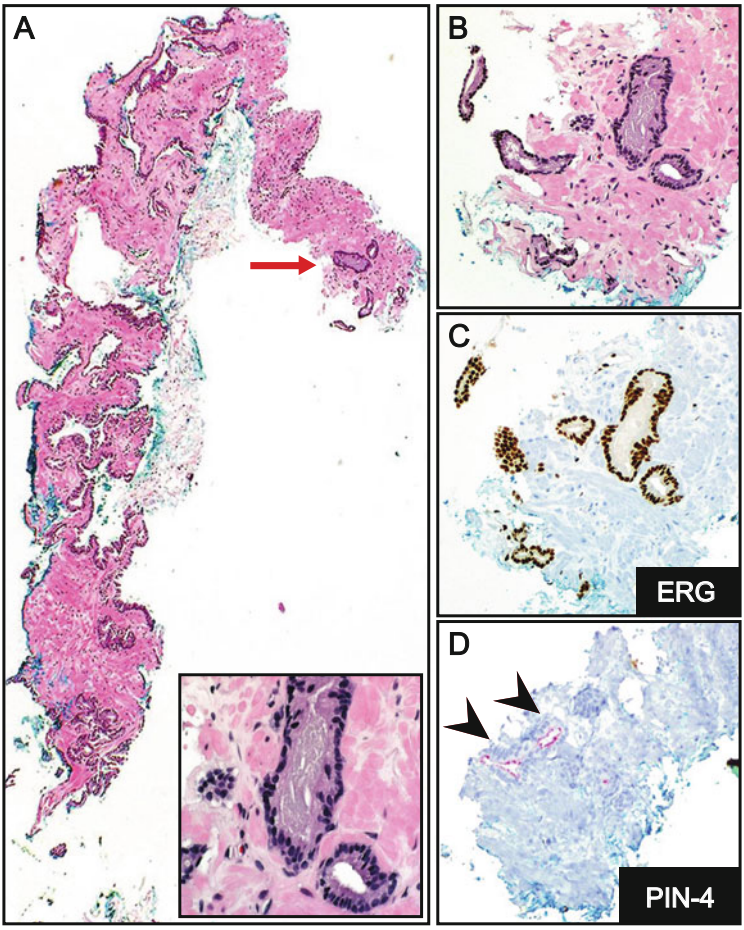

Fig. 17.2 ERG immunohistochemistry as a diagnostic adjunct in prostate cancer. **a** Low magnification view (40×) of a small suspicious focus on a single core (*red arrow*) from a 12 core prostate biopsy. Inset shows high power magnification (400×) highlighting the cytological and architectural atypia. **b** Medium power (200×) view of suspicious focus that was subsequently stained with **c** ERG as well as **d** PIN-4 cocktail (AMACR, red chromogen; p63 and CK903, brown chromogen). The atypical glands in question show strong nuclear staining with ERG and expression of AMACR; while basal cell markers are absent (*black arrowheads*); confirming the diagnosis of prostate cancer

ERG staining is positive in only 15 % of cases, these ERG positive glands are nearly always located adjacent to *TMPRSS2:ERG* rearranged invasive cancer [27, 45, 53, 54, 77, 124, 129, 130]. Therefore, in the context of a prostate biopsy with a challenging focus of atypical glands concerning for cancer, if the diagnosis of HGPIN can be excluded, ERG positivity indicates the presence of cancer and is more specific than AMACR/basal cell marker staining [29].

When HGPIN is encountered in isolation, the risk of carcinoma being present on subsequent prostate biopsy is around 25 %, which is not significantly different from the risk of cancer on repeat biopsy following a benign diagnosis [128]. No known clinical, demographic, or morphologic features exist than can reliably identify which men with isolated HGPIN are at high risk for cancer on follow up. Therefore, many urologists will not perform a repeat biopsy based solely the presence of HGPIN. The fact that ERG staining of HGPIN appears to only happen when directly adjacent to ERG positive carcinoma suggests that ERG positive isolated HGPIN on a prostate biopsy likely represents unsampled prostate cancer, and therefore may have use in risk stratification of patients with isolated HGPIN. To address this possibility, Park et al. evaluated prostate biopsies of 461 PSA screened patients with isolated HGPIN at baseline and at 12, 24, and 36 months of follow-up as part of a randomized, phase III clinical trial [131]. Fifty one patients (11.1 %) had ERG positive HGPIN, and this group was more likely to progress to prostate cancer on subsequent biopsy (53 % of ERG positive HGPIN patients developed cancer while only 35 % of ERG negative HGPIN patients developed cancer). These findings correlate with a Chinese cohort that also identified a higher incidence of cancer following ERG positive HGPIN [132]. These studies suggest that ERG positive isolated HGPIN warrants closer follow-up than ERG negative HGPIN and the fusion status can help drive clinical decision making.

17.8 Gene Fusions as a Basis for Molecular Subtyping and Targeted Therapy in Prostate Cancer

Several studies have now demonstrated that ETS positive and ETS negative prostate cancers are indeed distinct molecular subtypes based on mutation profiling and gene expression analysis [80, 83, 85, 133–135]. The ETS negative group of prostate cancers include tumours with outlier overexpression of *SPINK1* (encoding a peptidase inhibitor, found in ~10 % of prostate cancers) [136], loss or inactivation of *CHD1* (encoding a chromatin remodelling enzyme, found in ~5 % of prostate cancers) [137, 138], and/or mutations in *SPOP* (SPOP is involved in ubiquitin mediated degradation, found in 15 % of prostate cancers) [83, 139] as well as MAPK member fusions (discussed above, 1–2 % of prostate cancers). While these non-ETS molecular subgroupings are only found in ETS fusion negative tumour foci, they are not necessarily exclusive from one another [83, 84, 133]. These molecular subtypes have been used as a basis for predicting outcome as well as designing therapies.

The utility of *ETS* fusions in predicting prognosis is extremely complex, and has been recently reviewed [6]. Novel associations of *ERG* rearrangement status have recently been reported in regards to modulating the association between obesity and lethal prostate cancer [140], as well as progression while on active surveillance [141]. In multiple studies, combining *ETS* rearrangement status with status of the *PTEN* locus (an important tumour suppressor that is frequently inactivated in pros-

tate cancer) has been shown to have predictive value in various clinical situations, including PSA-screened, prostatectomy treated cohorts as well as incidentally discovered, conservatively managed patients; *ETS* rearrangement negative, *PTEN* wild type tumours were shown to have the best prognosis in multiple studies [142–145]. As the ability to comprehensively profile tumours becomes clinically feasible, molecular subtyping (utilising *ETS* fusion status; *CHD1*, *SPINK1*, and *SPOP* status; *RAF1*/*BRAF* fusion status; and important cancer genes such as *PTEN*, *TP53*, and *RB1*) has the potential to transition prostate cancer from a single clinically heterogeneous disease to a collection of distinct diseases defined by molecular features with separate risk factors, prognostic outcomes and therapeutic approaches.

Design of small molecule inhibitors to directly block transcription factors (like ETS family members) has proven to be challenging [146]; however, Pop et al. have recently reported the discovery of a compound that directly binds ETV1 and interferes with its transcriptional activity (Fig. 17.3) [147].

Other progress has been reported in targeting ETS fusions indirectly through interacting proteins such as PARP1 (poly (ADP)-ribose polymerase 1) (Fig. 17.3). PARP1 initiates the base excision DNA repair pathway activity and is necessary for maintaining ETS mediated oncogenic phenotypes in ETS fusion positive cell lines. Inhibition of PARP1 using the inhibitor olaparib in an ETS positive context leads to cell death via accumulation of DNA damage and leads to reduction tumours size in xenograft models [148]. Further, PARP1 inhibition reverses radiation resistance conferred to *ER*G overexpression [149]. Phase I trials of PARP1 inhibitors are encouraging [150–152]; and a multi-institutional phase II clinical trial for patients with metastatic, castrate resistant prostate cancer (stratified by ETS fusion status) evaluating PARP1 inhibition in the context of next generation anti-androgen treatment is underway (NCT01576172). Similar efforts to target additional ETS-interacting proteins such as PRKDC/DNA-PK [148] and USP9X [153] (Fig. 17.3) provide a possible avenue to apply precision medicine approaches to the *ETS* rearranged subtype of prostate cancer.

Directly targeting gene fusion products with small molecule inhibitors has proven to be an effective treatment strategy in certain cancers, as first demonstrated through the development of imatinib in chronic myeloid leukaemia [154]. The discovery of *RAF1*, *BRAF*, and *FGFR2* gene rearrangements in a small subset of prostate cancer identifies a possible analogous approach for patients with these tumours using approved or investigational inhibitors of RAF or FGFR kinase activity or downstream targets such as MEK (Fig. 17.3). Preclinical data shows the increased proliferation, invasion, and colony forming capabilities of RWPE cells overexpressing either *SLC45A3-BRAF* or *ESRP1-RAF1* are sensitive to MAPK/MEK pathway inhibition [23]. These results suggest that including these fusions in screening/molecular subtyping of prostate cancer can identify patients that can benefit from these drugs. Although rare within the context of prostate cancer (1–2 % as discussed above), the absolute number of patients that could benefit is much larger (thousands of men in the US each year) due to the overall high prevalence of prostate cancer.

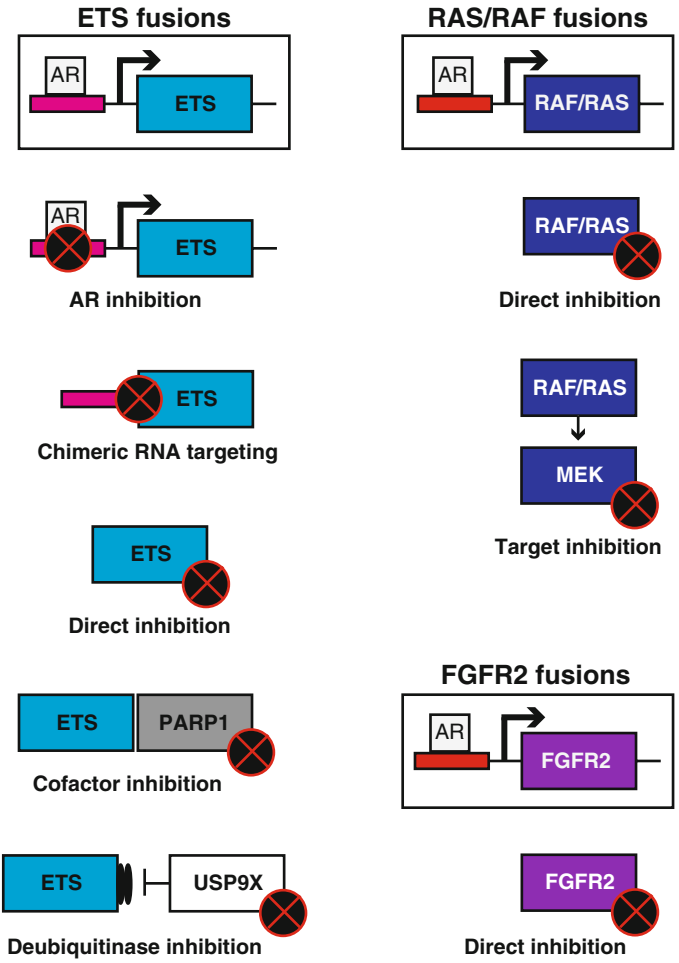

Fig. 17.3 Potential strategies to target gene fusions in prostate cancer. Numerous potential strategies have emerged for treating prostate cancer with ETS, RAS/RAF and FGFR2 fusion proteins. Androgen receptor inhibition can block production of androgen driven ETS, RAS/RAF and FGFR2 fusion proteins. Likewise, chimaeric mRNAs could be targeted by siRNA/shRNA. Historically, nuclear transcription factors, such as ETS gene fusions, have been considered undruggable. However, a small molecule capable of directly binding to ETV1 and disrupting transcription has recently been reported. Similarly, strategies to target required ERG cofactors, such as PARP1, and deubiquitinases, such as USP9X, also show promise. Targeting RAF/RAS fusion proteins may be possible by direct inhibitors or targeting obligate downstream targets (such as the RAF target MEK). Likewise, FGFR2 fusion proteins may be targetable by direct FGFR inhibitors

17.9 Summary and Future Directions

The discovery of gene fusions in prostate cancer initiated a fundamental shift in the understanding of prostate cancer enabling the molecular subtyping of patients and providing insight into the underlying biology of this disease. These gene fusions have led to improvements in early detection markers, risk stratification, and possible new therapies. We envision that in the near future, gene fusions and other key molecular alterations will be incorporated into routine clinical practice to provide more individualised therapy for patients with prostate cancer.

17.10 Competing Interests

The University of Michigan has been issued a patent on the detection of *ETS* gene fusions in prostate cancer, on which S.A.T. is a co-inventor. The University of Michigan licensed the diagnostic field of use to Gen-Probe, Inc., who has sublicensed some rights to Ventana/Roche. S.A.T. serves as a consultant to, and has received honoraria from, Ventana/Roche.

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References

1. Golub TR et al (1999) Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science 286:531–537
2. Ramaswamy S et al (2001) Multiclass cancer diagnosis using tumor gene expression signatures. Proc Natl Acad Sci U S A 98:15149–15154
3. Rubin MA et al (2002) alpha-Methylacyl coenzyme A racemase as a tissue biomarker for prostate cancer. JAMA 287:1662–1670
4. Van't Veer LJ et al (2002) Gene expression profiling predicts clinical outcome of breast cancer. Nature 415:530–536
5. Kumar-Sinha C, Tomlins SA, Chinnaiyan AM (2008) Recurrent gene fusions in prostate cancer. Nat Rev Cancer 8:497–511
6. Brenner JC, Chinnaiyan AM, Tomlins SA (2013) Fusion genes in prostate cancer. In: Tindall DJ (ed) Prostate cancer: biochemistry, molecular biology and genetics, Protein Reviews. Springer, New York, pp 139–183
7. Tomlins SA et al (2005) Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science 310:644–648
8. Tomlins SA et al (2009) ETS gene fusions in prostate cancer: from discovery to daily clinical practice. Eur Urol 56:275–286

9. Tomlins SA et al (2007) Distinct classes of chromosomal rearrangements create oncogenic ETS gene fusions in prostate cancer. Nature 448:595–599
10. Tomlins SA et al (2006) TMPRSS2:ETV4 gene fusions define a third molecular subtype of prostate cancer. Cancer Res 66:3396–3400
11. Helgeson BE et al (2008) Characterization of TMPRSS2:ETV5 and SLC45A3:ETV5 gene fusions in prostate cancer. Cancer Res 68:73–80
12. Rickman DS et al (2009) SLC45A3-ELK4 is a novel and frequent erythroblast transformation-specific fusion transcript in prostate cancer. Cancer Res 69:2734–2738
13. Paulo P et al (2011) FLI1 is a novel ETS transcription factor involved in gene fusions in prostate cancer. Genes Chromosom Cancer 51:240–249
14. Afar DE et al (2001) Catalytic cleavage of the androgen-regulated TMPRSS2 protease results in its secretion by prostate and prostate cancer epithelia. Cancer Res 61:1686–1692
15. Han B et al (2008) A fluorescence in situ hybridization screen for E26 transformation-specific aberrations: identification of DDX5-ETV4 fusion protein in prostate cancer. Cancer Res 68:7629–7637
16. Hermans KG et al (2008) Two unique novel prostate-specific and androgen-regulated fusion partners of ETV4 in prostate cancer. Cancer Res 68:3094–3098
17. Mehra R et al (2008) Characterization of TMPRSS2-ETS gene aberrations in androgen-independent metastatic prostate cancer. Cancer Res 68:3584–3590
18. Barros-Silva JD et al (2013) Novel 5′ fusion partners of ETV1 and ETV4 in prostate cancer. Neoplasia 15:720–726
19. Maher CA et al (2009) Transcriptome sequencing to detect gene fusions in cancer. Nature 458:97–101
20. Nacu S et al (2011) Deep RNA sequencing analysis of readthrough gene fusions in human prostate adenocarcinoma and reference samples. BMC Med Genomics 4:11
21. Zhang Y et al (2012) Chimeric transcript generated by cis-splicing of adjacent genes regulates prostate cancer cell proliferation. Cancer Discov 2:598–607
22. Maher CA et al (2009) Chimeric transcript discovery by paired-end transcriptome sequencing. Proc Natl Acad Sci U S A 106:12353–12358
23. Palanisamy N et al (2010) Rearrangements of the RAF kinase pathway in prostate cancer, gastric cancer and melanoma. Nat Med 16:793–798
24. Lin B et al (1999) Prostate-localized and androgen-regulated expression of the membrane-bound serine protease TMPRSS2. Cancer Res 59:4180–4184
25. Vaarala MH, Porvari K, Kyllonen A, Lukkarinen O, Vihko P (2001) The TMPRSS2 gene encoding transmembrane serine protease is overexpressed in a majority of prostate cancer patients: detection of mutated TMPRSS2 form in a case of aggressive disease. Int J Cancer 94:705–710
26. Falzarano SM et al (2011) ERG gene rearrangement status in prostate cancer detected by immunohistochemistry. Virchows Arch 459:441–447
27. Park K et al (2010) Antibody-based detection of ERG rearrangement-positive prostate cancer. Neoplasia 12:590–598
28. Perner S et al (2013) Loss of SLC45A3 protein (prostein) expression in prostate cancer is associated with SLC45A3-ERG gene rearrangement and an unfavorable clinical course. Int J Cancer 132(4):807–12
29. Tomlins SA, Palanisamy N, Siddiqui J, Chinnaiyan AM, Kunju LP (2012) Antibody-based detection of ERG rearrangements in prostate core biopsies, including diagnostically challenging cases: ERG staining in prostate core biopsies. Arch Pathol Lab Med 136:935–946
30. Cerveira N et al (2006) TMPRSS2-ERG gene fusion causing ERG overexpression precedes chromosome copy number changes in prostate carcinomas and paired HGPIN lesions. Neoplasia 8:826–832
31. Clark J et al (2007) Diversity of TMPRSS2-ERG fusion transcripts in the human prostate. Oncogene 26:2667–2673

32. Hermans KG et al (2009) Overexpression of prostate-specific TMPRSS2(exon 0)-ERG fusion transcripts corresponds with favorable prognosis of prostate cancer. Clin Cancer Res 15:6398–6403
33. Hermans KG et al (2006) TMPRSS2:ERG fusion by translocation or interstitial deletion is highly relevant in androgen-dependent prostate cancer, but is bypassed in late-stage androgen receptor-negative prostate cancer. Cancer Res 66:10658–10663
34. Iljin K et al (2006) TMPRSS2 fusions with oncogenic ETS factors in prostate cancer involve unbalanced genomic rearrangements and are associated with HDAC1 and epigenetic reprogramming. Cancer Res 66:10242–10246
35. Lapointe J et al (2007) A variant TMPRSS2 isoform and ERG fusion product in prostate cancer with implications for molecular diagnosis. Mod Pathol 20:467–473
36. Rajput AB et al (2007) Frequency of the TMPRSS2:ERG gene fusion is increased in moderate to poorly differentiated prostate cancers. J Clin Pathol 60:1238–1243
37. Soller MJ et al (2006) Confirmation of the high frequency of the TMPRSS2/ERG fusion gene in prostate cancer. Genes Chromosom Cancer 45:717–719
38. Wang J, Cai Y, Ren C, Ittmann M (2006) Expression of variant TMPRSS2/ERG fusion messenger RNAs is associated with aggressive prostate cancer. Cancer Res 66:8347–8351
39. Yoshimoto M et al (2006) Three-color FISH analysis of TMPRSS2/ERG fusions in prostate cancer indicates that genomic microdeletion of chromosome 21 is associated with rearrangement. Neoplasia 8:465–469
40. Hu Y et al (2008) Delineation of TMPRSS2-ERG splice variants in prostate cancer. Clin Cancer Res 14:4719–4725
41. Wang J et al (2011) Activation of NF-{kappa}B by TMPRSS2/ERG Fusion Isoforms through Toll-Like Receptor-4. Cancer Res 71:1325–1333
42. Wang J et al (2008) Pleiotropic biological activities of alternatively spliced TMPRSS2/ERG fusion gene transcripts. Cancer Res 68:8516–8524
43. Pettersson A et al (2012) The TMPRSS2:ERG rearrangement, ERG expression, and prostate cancer outcomes: a cohort study and meta-analysis. Cancer Epidemiol Biomarkers Prev 21:1497–1509
44. Mehra R et al (2007) Comprehensive assessment of TMPRSS2 and ETS family gene aberrations in clinically localized prostate cancer. Mod Pathol 20:538–544
45. Furusato B et al (2010) ERG oncoprotein expression in prostate cancer: clonal progression of ERG-positive tumor cells and potential for ERG-based stratification. Prostate Cancer Prostatic Dis 13:228–237
46. Attard G et al (2008) Duplication of the fusion of TMPRSS2 to ERG sequences identifies fatal human prostate cancer. Oncogene 27:253–263
47. Braun M et al (2012) ERG protein expression and genomic rearrangement status in primary and metastatic prostate cancer-a comparative study of two monoclonal antibodies. Prostate Cancer Prostatic Dis 15(2):165–169
48. Demichelis F et al (2007) TMPRSS2:ERG gene fusion associated with lethal prostate cancer in a watchful waiting cohort. Oncogene 26:4596–4599
49. Falzarano SM et al (2010) ERG rearrangement is present in a subset of transition zone prostatic tumors. Mod Pathol 23:1499–1506
50. FitzGerald LM et al (2008) Association of TMPRSS2-ERG gene fusion with clinical characteristics and outcomes: results from a population-based study of prostate cancer. BMC Cancer 8:230
51. Guo CC, Zuo G, Cao D, Troncoso P, Czerniak BA (2009) Prostate cancer of transition zone origin lacks TMPRSS2-ERG gene fusion. Mod Pathol 22:866–871
52. Liu S et al (2011) Detection of ERG gene rearrangements and PTEN deletions in unsuspected prostate cancer of the transition zone. Cancer Biol Ther 11:562–566
53. Mosquera JM et al (2009) Prevalence of TMPRSS2-ERG fusion prostate cancer among men undergoing prostate biopsy in the United States. Clin Cancer Res 15:4706–4711

54. van Leenders GJ et al (2011) Antibody EPR3864 is specific for ERG genomic fusions in prostate cancer: implications for pathological practice. Mod Pathol 24:1128–1138
55. Yaskiv O et al (2011) The utility of ERG/P63 double immunohistochemical staining in the diagnosis of limited cancer in prostate needle biopsies. Am J Surg Pathol 35:1062–1068
56. Magi-Galluzzi C et al (2010) TMPRSS2-ERG gene fusion prevalence and class are significantly different in prostate cancer of Caucasian, African-American and Japanese patients. Prostate 71(5):489–497
57. Mao X et al (2010) Distinct genomic alterations in prostate cancers in Chinese and Western populations suggest alternative pathways of prostate carcinogenesis. Cancer Res 70:5207–5212
58. Miyagi Y et al (2010) ETS family-associated gene fusions in Japanese prostate cancer: analysis of 194 radical prostatectomy samples. Mod Pathol 23:1492–1498
59. Raymundo EM et al (2014) Increased association of the ERG oncoprotein expression in advanced stages of prostate cancer in Filipinos. Prostate 74(11):1079–1085
60. Dong J, Xiao L, Sheng L, Xu J, Sun ZQ (2014) TMPRSS2:ETS fusions and clinicopathologic characteristics of prostate cancer patients from Eastern China. Asian Pac J Cancer Prev 15:3099–3103
61. Zhau HE, Li Q, Chung LW (2013) Interracial differences in prostate cancer progression among patients from the United States, China and Japan. Asian J Androl 15:705–707
62. Wang JJ et al (2012) Fusion between TMPRSS2 and ETS family members (ERG, ETV1, ETV4) in prostate cancers from northern China. Asian Pac J Cancer Prev 13:4935–4938
63. Qi M et al (2014) ERG rearrangement is associated with prostate cancer-related death in Chinese prostate cancer patients. PLoS One 9:e84959
64. Schaefer G et al (2013) Distinct ERG rearrangement prevalence in prostate cancer: higher frequency in young age and in low PSA prostate cancer. Prostate Cancer Prostatic Dis 16(2):132–138
65. Weischenfeldt J et al (2013) Integrative genomic analyses reveal an androgen-driven somatic alteration landscape in early-onset prostate cancer. Cancer Cell 23:159–170
66. Korenchuk S et al (2001) VCaP, a cell-based model system of human prostate cancer. In Vivo 15:163–168
67. Tomlins SA et al (2008) Role of the TMPRSS2-ERG gene fusion in prostate cancer. Neoplasia 10:177–188
68. Hollenhorst PC, Paul L, Ferris MW, Graves BJ (2011) The ETS gene ETV4 is required for anchorage-independent growth and a cell proliferation gene expression program in PC3 prostate cells. Genes Cancer 1:1044–1052
69. Hollenhorst PC et al (2011) Oncogenic ETS proteins mimic activated RAS/MAPK signaling in prostate cells. Genes Dev 25:2147–2157
70. Carver BS et al (2009) ETS rearrangements and prostate cancer initiation. Nature 457:E1, discussion E2-3
71. Casey OM et al (2012) TMPRSS2- driven ERG expression in vivo increases self-renewal and maintains expression in a castration resistant subpopulation. PLoS One 7:e41668
72. King JC et al (2009) Cooperativity of TMPRSS2-ERG with PI3-kinase pathway activation in prostate oncogenesis. Nat Genet 41:524–526
73. Klezovitch O et al (2008) A causal role for ERG in neoplastic transformation of prostate epithelium. Proc Natl Acad Sci U S A 105:2105–2110
74. Zong Y et al (2009) ETS family transcription factors collaborate with alternative signaling pathways to induce carcinoma from adult murine prostate cells. Proc Natl Acad Sci U S A 106:12465–12470
75. Chen Y et al (2013) ETS factors reprogram the androgen receptor cistrome and prime prostate tumorigenesis in response to PTEN loss. Nat Med 19:1023–1029
76. Baena E et al (2013) ETV1 directs androgen metabolism and confers aggressive prostate cancer in targeted mice and patients. Genes Dev 27:683–698

77. Carver BS et al (2009) Aberrant ERG expression cooperates with loss of PTEN to promote cancer progression in the prostate. Nat Genet 41:619–624
78. Mosquera JM et al (2008) Characterization of TMPRSS2-ERG fusion high-grade prostatic intraepithelial neoplasia and potential clinical implications. Clin Cancer Res 14:3380–3385
79. Perner S et al (2007) TMPRSS2-ERG fusion prostate cancer: an early molecular event associated with invasion. Am J Surg Pathol 31:882–888
80. Tomlins SA et al (2007) Integrative molecular concept modeling of prostate cancer progression. Nat Genet 39:41–51
81. Bakin RE, Gioeli D, Sikes RA, Bissonette EA, Weber MJ (2003) Constitutive activation of the Ras/mitogen-activated protein kinase signaling pathway promotes androgen hypersensitivity in LNCaP prostate cancer cells. Cancer Res 63:1981–1989
82. Bakin RE, Gioeli D, Bissonette EA, Weber MJ (2003) Attenuation of Ras signaling restores androgen sensitivity to hormone-refractory C4-2 prostate cancer cells. Cancer Res 63:1975–1980
83. Barbieri CE et al (2012) Exome sequencing identifies recurrent SPOP, FOXA1 and MED12 mutations in prostate cancer. Nat Genet 44:685–689
84. Grasso CS et al (2012) The mutational landscape of lethal castration-resistant prostate cancer. Nature 487:239–243
85. Taylor BS et al (2010) Integrative genomic profiling of human prostate cancer. Cancer Cell 18:11–22
86. Esgueva R et al (2010) Prevalence of TMPRSS2-ERG and SLC45A3-ERG gene fusions in a large prostatectomy cohort. Mod Pathol 23:539–546
87. Han B et al (2009) Characterization of ETS gene aberrations in select histologic variants of prostate carcinoma. Mod Pathol 22:1176–1185
88. Beltran H et al (2013) Targeted next-generation sequencing of advanced prostate cancer identifies potential therapeutic targets and disease heterogeneity. Eur Urol 63(5):920–926
89. Ren G et al (2012) Identification of frequent BRAF copy number gain and alterations of RAF genes in Chinese prostate cancer. Genes Chromosom Cancer 51(11):1014–1023
90. Wang XS et al (2011) Characterization of KRAS rearrangements in metastatic prostate cancer. Cancer Discov 1:35–43
91. Goueli BS, Janknecht R (2004) Upregulation of the catalytic telomerase subunit by the transcription factor ER81 and oncogenic HER2/Neu, Ras, or Raf. Mol Cell Biol 24:25–35
92. Bosc DG, Janknecht R (2002) Regulation of Her2/neu promoter activity by the ETS transcription factor, ER81. J Cell Biochem 86:174–183
93. Janknecht R, Monte D, Baert JL, de Launoit Y (1996) The ETS-related transcription factor ERM is a nuclear target of signaling cascades involving MAPK and PKA. Oncogene 13:1745–1754
94. Janknecht R (1996) Analysis of the ERK-stimulated ETS transcription factor ER81. Mol Cell Biol 16:1550–1556
95. Ren S et al (2012) RNA-seq analysis of prostate cancer in the Chinese population identifies recurrent gene fusions, cancer-associated long noncoding RNAs and aberrant alternative splicings. Cell Res 22:806–821
96. Prensner JR, Chinnaiyan AM (2011) The emergence of lncRNAs in cancer biology. Cancer Discov 1:391–407
97. Prensner JR et al (2011) Transcriptome sequencing across a prostate cancer cohort identifies PCAT-1, an unannotated lincRNA implicated in disease progression. Nat Biotechnol 29:742–749
98. Yang L et al (2013) lncRNA-dependent mechanisms of androgen-receptor-regulated gene activation programs. Nature 500:598–602
99. Kannan K et al (2011) Recurrent chimeric RNAs enriched in human prostate cancer identified by deep sequencing. Proc Natl Acad Sci U S A 108:9172–9177
100. Roychowdhury S et al (2011) Personalized oncology through integrative high-throughput sequencing: a pilot study. Sci Transl Med 3:111–121

101. Wu YM et al (2013) Identification of targetable FGFR gene fusions in diverse cancers. Cancer Discov 3(6):636–647
102. Kelleher FC, O'Sullivan H, Smyth E, McDermott R, Viterbo A (2013) Fibroblast growth factor receptors, developmental corruption and malignant disease. Carcinogenesis 34:2198–2205
103. Glinsky GV, Glinskii AB, Stephenson AJ, Hoffman RM, Gerald WL (2004) Gene expression profiling predicts clinical outcome of prostate cancer. J Clin Invest 113:913–923
104. Rostad K et al (2009) TMPRSS2:ERG fusion transcripts in urine from prostate cancer patients correlate with a less favorable prognosis. Apmis 117:575–582
105. Nilsson J et al (2009) Prostate cancer-derived urine exosomes: a novel approach to biomarkers for prostate cancer. Br J Cancer 100:1603–1607
106. Hessels D et al (2007) Detection of TMPRSS2-ERG fusion transcripts and prostate cancer antigen 3 in urinary sediments may improve diagnosis of prostate cancer. Clin Cancer Res 13:5103–5108
107. Laxman B et al (2006) Noninvasive detection of TMPRSS2:ERG fusion transcripts in the urine of men with prostate cancer. Neoplasia 8:885–888
108. Laxman B et al (2008) A first-generation multiplex biomarker analysis of urine for the early detection of prostate cancer. Cancer Res 68:645–649
109. Cao DL et al (2011) A multiplex model of combining gene-based, protein-based, and metabolite-based with positive and negative markers in urine for the early diagnosis of prostate cancer. Prostate 71:700–710
110. Nguyen PN et al (2011) A panel of TMPRSS2:ERG fusion transcript markers for urine-based prostate cancer detection with high specificity and sensitivity. Eur Urol 59:407–414
111. Salami SS et al (2013) Combining urinary detection of TMPRSS2:ERG and PCA3 with serum PSA to predict diagnosis of prostate cancer. Urol Oncol 31(5):566–71
112. Tomlins SA et al (2011) Urine TMPRSS2:ERG fusion transcript stratifies prostate cancer risk in men with elevated serum PSA. Sci Transl Med 3:94ra72
113. Sabaliauskaite R et al (2012) Combined analysis of TMPRSS2-ERG and TERT for improved prognosis of biochemical recurrence in prostate cancer. Genes Chromosom Cancer 51:781–791
114. Cornu JN et al (2012) Urine TMPRSS2:ERG fusion transcript integrated with PCA3 score, genotyping, and biological features are correlated to the results of prostatic biopsies in men at risk of prostate cancer. Prostate 73:242–249
115. Leyten GH et al (2014) Prospective multicentre evaluation of PCA3 and TMPRSS2-ERG gene fusions as diagnostic and prognostic urinary biomarkers for prostate cancer. Eur Urol 65:534–542
116. Groskopf J et al (2006) APTIMA PCA3 molecular urine test: development of a method to aid in the diagnosis of prostate cancer. Clin Chem 52:1089–1095
117. Lin DW et al (2013) Urinary TMPRSS2:ERG and PCA3 in an active surveillance cohort: results from a baseline analysis in the Canary Prostate Active Surveillance Study. Clin Cancer Res 19:2442–2450
118. Young A et al (2012) Correlation of urine TMPRSS2:ERG and PCA3 to ERG+ and total prostate cancer burden. Am J Clin Pathol 138:685–696
119. Beach R et al (2002) P504S immunohistochemical detection in 405 prostatic specimens including 376 18-gauge needle biopsies. Am J Surg Pathol 26:1588–1596
120. Epstein JI, Netto GJ (2008) Biopsy interpretation of the prostate. Wolters Kluwer Health/ Lippincott Williams & Wilkins, Philadelphia, x, 358 p
121. Kunju LP, Rubin MA, Chinnaiyan AM, Shah RB (2003) Diagnostic usefulness of monoclonal antibody P504S in the workup of atypical prostatic glandular proliferations. Am J Clin Pathol 120:737–745
122. Przybycin CG, Kunju LP, Wu AJ, Shah RB (2008) Partial atrophy in prostate needle biopsies: a detailed analysis of its morphology, immunophenotype, and cellular kinetics. Am J Surg Pathol 32:58–64

123. Chaux A et al (2011) Immunohistochemistry for ERG expression as a surrogate for TMPRSS2-ERG fusion detection in prostatic adenocarcinomas. Am J Surg Pathol 35:1014–1020
124. He H et al (2011) The diagnostic utility of novel immunohistochemical marker ERG in the workup of prostate biopsies with “atypical glands suspicious for cancer”. Am J Surg Pathol 35:608–614
125. Hoogland AM et al (2012) ERG immunohistochemistry is not predictive for PSA recurrence, local recurrence or overall survival after radical prostatectomy for prostate cancer. Mod Pathol 25(3):471–9
126. Kron K et al (2012) Correlation of ERG expression and DNA methylation biomarkers with adverse clinicopathologic features of prostate cancer. Clin Cancer Res 18:2896–2904
127. Epstein JI (2009) Precursor lesions to prostatic adenocarcinoma. Virchows Arch 454:1–16
128. Epstein JI, Herawi M (2006) Prostate needle biopsies containing prostatic intraepithelial neoplasia or atypical foci suspicious for carcinoma: implications for patient care. J Urol 175:820–834
129. Han B et al (2009) Fluorescence in situ hybridization study shows association of PTEN deletion with ERG rearrangement during prostate cancer progression. Mod Pathol 22:1083–1093
130. Minner S et al (2013) Marked heterogeneity of ERG expression in large primary prostate cancers. Mod Pathol 26:106–116
131. Park K et al (2014) TMPRSS2:ERG gene fusion predicts subsequent detection of prostate cancer in patients with high-grade prostatic intraepithelial neoplasia. J Clin Oncol 32:206–211
132. Gao X et al (2012) ERG rearrangement for predicting subsequent cancer diagnosis in high-grade prostatic intraepithelial neoplasia and lymph node metastasis. Clin Cancer Res 18:4163–4172
133. Baca SC et al (2013) Punctuated evolution of prostate cancer genomes. Cell 153:666–677
134. Jhavar S et al (2009) Integration of ERG gene mapping and gene-expression profiling identifies distinct categories of human prostate cancer. BJU Int 103:1256–1269
135. Setlur SR et al (2008) Estrogen-dependent signaling in a molecularly distinct subclass of aggressive prostate cancer. J Natl Cancer Inst 100:815–825
136. Tomlins SA et al (2008) The role of SPINK1 in ETS rearrangement-negative prostate cancers. Cancer Cell 13:519–528
137. Huang S et al (2012) Recurrent deletion of CHD1 in prostate cancer with relevance to cell invasiveness. Oncogene 31:4164–4170
138. Liu W et al (2012) Identification of novel CHD1-associated collaborative alterations of genomic structure and functional assessment of CHD1 in prostate cancer. Oncogene 31:3939–3948
139. Blattner M et al (2014) SPOP mutations in prostate cancer across demographically diverse patient cohorts. Neoplasia 16:14–20
140. Pettersson A et al (2013) Modification of the association between obesity and lethal prostate cancer by TMPRSS2:ERG. J Natl Cancer Inst 105:1881–1890
141. Berg KD et al (2014) ERG protein expression in diagnostic specimens is associated with increased risk of progression during active surveillance for prostate cancer. Eur Urol 66(5):851–860
142. Krohn A et al (2012) Genomic deletion of PTEN is associated with tumor progression and early PSA recurrence in ERG fusion-positive and fusion-negative prostate cancer. Am J Pathol 181:401–412
143. Reid AH et al (2010) Molecular characterisation of ERG, ETV1 and PTEN gene loci identifies patients at low and high risk of death from prostate cancer. Br J Cancer 102:678–684
144. Yoshimoto M et al (2008) Absence of TMPRSS2:ERG fusions and PTEN losses in prostate cancer is associated with a favorable outcome. Mod Pathol 21:1451–1460

145. Fontugne J et al (2014) Recurrent prostate cancer genomic alterations predict response to brachytherapy treatment. Cancer Epidemiol Biomarkers Prev 23:594–600
146. Darnell JE Jr (2002) Transcription factors as targets for cancer therapy. Nat Rev Cancer 2:740–749
147. Pop MS et al (2014) A small molecule that binds and inhibits the ETV1 transcription factor oncoprotein. Mol Cancer Ther 13:1492–1502
148. Brenner JC et al (2011) Mechanistic rationale for inhibition of poly(ADP-ribose) polymerase in ETS gene fusion-positive prostate cancer. Cancer Cell 19:664–678
149. Han S et al (2013) Targeted radiosensitization of ETS fusion-positive prostate cancer through PARP1 inhibition. Neoplasia 15:1207–1217
150. Fong PC et al (2009) Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. N Engl J Med 361:123–134
151. Sandhu SK et al (2013) Poly (ADP-ribose) polymerase (PARP) inhibitors for the treatment of advanced germline BRCA2 mutant prostate cancer. Ann Oncol 24:1416–1418
152. Sandhu SK et al (2013) The poly(ADP-ribose) polymerase inhibitor niraparib (MK4827) in BRCA mutation carriers and patients with sporadic cancer: a phase 1 dose-escalation trial. Lancet Oncol 14:882–892
153. Wang S et al (2014) Ablation of the oncogenic transcription factor ERG by deubiquitinase inhibition in prostate cancer. Proc Natl Acad Sci U S A 111:4251–4256
154. Druker BJ (2008) Translation of the Philadelphia chromosome into therapy for CML. Blood 112:4808–4817

Chapter 18
Chromosomal Translocations in Lung Cancer

Hiroyuki Mano

Contents

18.1 Introduction 404
18.2 *ALK* Fusions 406
18.2.1 *EML4-ALK* and Other *ALK* Fusions 406
18.2.2 Clinicopathologic Features 407
18.2.3 Crizotinib 407
18.2.4 Resistance to Crizotinib 408
18.2.5 Second-Generation ALK Inhibitors 409
18.3 *ROS1* Fusions 410
18.3.1 Types of *ROS1* Fusion Proteins 410
18.3.2 Targeted Therapies 410
18.4 *RET* Fusions 410
18.4.1 *KIF5B-RET* and Other *RET* Fusions 410
18.4.2 Targeted Therapies 411
18.5 Other Gene Fusions in Lung Cancer 411
18.5.1 *FGFR* Family Fusions 411
18.5.2 *NTRK1* Fusions 412
18.5.3 Other Fusions 412
18.6 Concluding Remarks 413
References 413

Abstract Discovery of the *EML4-ALK* fusion-type oncogene had triggered extensive search for tyrosine kinase fusions in lung cancer (and other cancer types), resulting in the identification of various *ALK*-, *ROS1*-, *RET*-, *FGFR1/2/3*- and other kinase gene-fusions. Marked therapeutic efficacy of ALK inhibitors was swiftly confirmed in patients with *ALK*-rearranged tumours, and some of the compounds are already brought to the clinics. Likewise, specific inhibitors for each fusion kinase are currently under clinical investigation. Lung cancer is thus one of the tumour types that identification of fusion genes has most contributed to improve directly the treatment strategies for.

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18.1 Introduction

Lung cancer remains the leading cause of cancer-related deaths worldwide, with ~1.4 million individuals dying of this disorder annually [1]. On the basis of pathological characteristics as well as the clinical response to chemo- and radiotherapy, lung cancer is subdivided into small cell lung cancer (SCLC) and non–small cell lung cancer (NSCLC), the latter of which includes squamous cell carcinoma, adenocarcinoma, and large cell carcinoma.

Only a decade or so ago, essentially a single driver oncogene was known for lung cancer—mutated *KRAS*. Recently, however, remarkable progress has been made in our understanding of the genetic alterations that underlie, and in the development of new treatment strategies for, lung cancer (mainly NSCLC). First, somatic activating mutations of the epidermal growth factor receptor (EGFR) were identified in ~10–40 % of individuals with lung adenocarcinoma, and specific inhibitors of EGFR have proved to be effective for the treatment of such individuals [2]. And second, *EML4-ALK* was identified as a fusion oncogene in ~4–5 % of NSCLC cases [3]. This was the first recurrent fusion gene shown to be associated with lung cancer, and its discovery, together with that of *TMPRSS2-ERG* in prostate cancer, revealed the importance of fusion-type oncogenes in epithelial tumours. The identification of *EML4-ALK* led to the rapid development of ALK inhibitors as potential therapeutic agents, with one such drug, crizotinib, having been approved by the U.S. Food and Drug Administration (FDA) only 4 years after discovery of the fusion gene. *EML4-ALK* encodes a constitutively active protein tyrosine kinase (PTK) that plays a pivotal role in carcinogenesis. Lung cancer cells harbouring this PTK are dependent on its activity, such that inhibition of this activity is highly effective in inducing tumour cell death.

The discovery of *EML4-ALK* and the clinical success of ALK inhibitors have become a paradigm for the development and implementation of effective molecularly targeted therapy for cancer. The approach thus requires (1) the identification of an essential growth driver, (2) the development of specific inhibitors, (3) the simultaneous development of companion diagnostics, and (4) the treatment of only those patients who harbour the targeted genetic alteration. Since the identification of *EML4-ALK*, many additional gene fusions have been detected in lung cancer (Table 18.1), with similar clinical application of these discoveries now being under way.

Table 18.1 Fusion genes in lung cancer

Fusion gene	Frequency	Reference
EML4-ALK	4–5 %	[3]
KIF5B-ALK	<1 %	[9]
TFG-ALK	–	[10]
STRN-ALK	–	[11]
KLC1-ALK	–	[12]
HIP1-ALK	–	[13]
CD74-ROS1	1–3 %	[10]
SLC34A2-ROS1		[10]
SDC4-ROS1		[27]
TPM3-ROS1		[27]
EZR-ROS1		[27]
LRIG3-ROS1		[27]
CCDC6-ROS1		[28]
GOPC-ROS1		[29]
KDELR2-ROS1		[30]
KIF5B-RET	1–2 %	[27, 33–35]
CCDC6-RET		[27]
NCOA4-RET		[36]
TRIM33-RET		[37]
FGFR3-TACC3	–	[11, 40]
FGFR2-CCAR2	–	[40]
FGFR2-CIT	–	[28]
BAG4-FGFR1	–	[40]
MPRIP-NTRK1	–	[41]
CD74-NTRK1	–	[41]
AXL-MBIP	–	[28]
SCAF11-PDGFRA	–	[28]
MAP4K3-PRKCE	–	[28]
BCAS3-MAP3K3	–	[28]
ERBB2IP-MAST4	–	[28]
MARK4-ERCC2	–	[42]
KRAS-CDH13	–	[28]
APLP2-TNFSF11	–	[28]
ZFYVE9-CGA	–	[28]

(continued)

Table 18.1 (continued)

Fusion gene	Frequency	Reference
TPD52L1-TRMT11	–	[28]
RHPN2-PEPD	–	[42]
SIRT2-NPHS1	–	[42]
NIPAL3-ATAD3B	–	[42]
UBFD1-CDH11	–	[42]
GRHL2-PTPN12	–	[42]
GLE1-CCBL1	–	[42]

Only in-frame fusion genes are listed

18.2 *ALK* Fusions

18.2.1 EML4-ALK *and Other* ALK *Fusions*

The *EML4-ALK* fusion-type oncogene was identified with a functional screening approach [3]. The *EML4* (echinoderm microtubule-associated protein–like 4) and *ALK* (anaplastic lymphoma kinase) genes are both located on the short arm of human chromosome 2 but have opposite orientations, with a small inversion involving the two loci, inv(2)(p21p23.2), being responsible for fusion of the two genes (Fig. 18.1).

The EML4 portion of the fusion protein contains a coiled-coil domain essential for oligomerization, whereas the ALK portion contains the catalytic domain. The constitutive oligomerization of EML4-ALK mediated by the coiled-coil domain thus results in constitutive PTK activation and thereby confers transforming ability. Most other kinase fusions also include a partner protein that contains an oligomerization motif.

The marked oncogenic activity of EML4-ALK was demonstrated in transgenic mice. The promoter of the surfactant protein C gene was used to drive the expression of *EML4-ALK* specifically in bronchial alveolar cells. The transgenic mice were found to develop hundreds of adenocarcinoma nodules soon after birth, and treatment of the animals with ALK inhibitors resulted in eradication of the tumour nodules [4]. Similar data were obtained with another transgenic mouse model, with the EML4-ALK–positive tumours also being shown to be sensitive to a heat shock protein 70 (HSP70) inhibitor [5].

The fusion points of *ALK* are located exclusively within intron 19, resulting in fusion to exon 20 of *ALK* in the corresponding mRNAs. On the other hand, various fusion points of *EML4* have been identified. Given that the coiled-coil domain is present at the amino terminus of EML4 (corresponding to exon 2), any in-frame fusions that include *EML4* exon 2 will likely generate an oncogenic kinase. Indeed, whereas the originally discovered form of *EML4-ALK* was the result of ligation between intron 13 of *EML4* and intron 19 of *ALK*, many other *EML4* introns are potential fusion sites. Although introns 6 and 13 of *EML4* are the "hotspots" for fusion, all variants should be screened for clinically, given that they are all sensitive to ALK inhibitors [6–8].

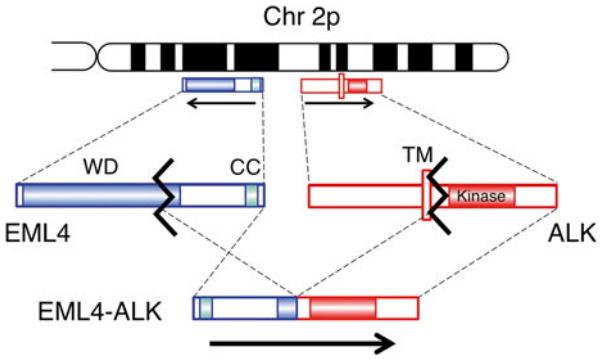

Fig. 18.1 A small inversion within the short arm of human chromosome 2 generates the *EML4-ALK* fusion gene. The *EML4* and *ALK* genes have opposite orientations, but a small inversion, inv(2)(p21p23.2), results in their fusion in the same direction. *WD* WD domain, *CC* coiled-coil domain, *TM* transmembrane domain

Other *ALK* fusions shown to result from chromosome rearrangement in lung cancer include *KIF5B-ALK* [9], *TFG-ALK* [10], *STRN-ALK* [11], *KLC1-ALK* [12], and *HIP1-ALK* [13]. Whereas *KIF5B-ALK* was confirmed to be recurrent, the other fusions have as yet been described only in case reports.

18.2.2 Clinicopathologic Features

Several large-scale screenings for *ALK* rearrangement have revealed clinical characteristics of lung cancer positive for such rearrangement [8, 14, 15]. Individuals with lung cancer positive for *EML4-ALK* tend to be never- or light smokers, and their tumours are almost exclusively adenocarcinoma (with rare cases of squamous cell carcinoma). They also tend to be relatively young at the time of tumour detection, with a mean age of diagnosis of around 50 years. Importantly, *EML4-ALK* and *EGFR* mutations appear to be mutually exclusive in the vast majority of tumours. Lung adenocarcinoma negative for *EGFR* mutations is thus a good target for studies of *EML4-ALK*. Pathologically, *EML4-ALK*–positive tumours often manifest a mucinous cribriform or signet-ring cell pattern, although other pathological subtypes are also observed [16].

18.2.3 Crizotinib

Crizotinib (previously known as PF-2341066) was the first compound to enter clinical trials for *EML4-ALK*–positive NSCLC. It is an orally available competitive inhibitor of ATP binding to the PTKs MET and ALK [17], with median inhibitory concentrations of 5–20 nM and 24 nM, respectively. Clinical trials of crizotinib

have been notable for two characteristics: (1) the companion diagnostic test—a break-apart fluorescence in situ hybridization (FISH) assay for detection of *ALK* locus rearrangement—was simultaneously developed; and (2) with the application of this assay, only NSCLC patients with *ALK* fusions were enrolled. These characteristics made it possible to demonstrate the marked efficacy of crizotinib in an unusually short period of time.

The report of the first crizotinib trial revealed an overall response rate of 57 %, with an estimated probability of 6-month progression-free survival of 72 % [14]. Major adverse events included visual disturbance, nausea, vomiting, and diarrhoea, and severe events included liver toxicity and pneumonitis. On the basis of these data (without the results of a phase 3 trial), the U.S. FDA swiftly approved crizotinib in August 2011. A subsequent phase 3 study revealed that crizotinib was superior to the standard chemotherapies with regard to both response rate and progression-free survival [18].

18.2.4 Resistance to Crizotinib

Whereas crizotinib was found to be highly effective for the treatment of patients with *ALK* rearrangement–positive tumours, most such treated patients eventually manifest disease relapse. The first study to decipher the resistance mechanisms was based on analysis of pleural effusion in a patient who underwent relapse after ~6 months of successful crizotinib treatment [19]. Deep sequencing with a next-generation sequencer revealed two point mutations—C1156Y and L1196M—within the kinase domain of ALK only in the crizotinib-tolerant tumour (Fig. 18.2). Of

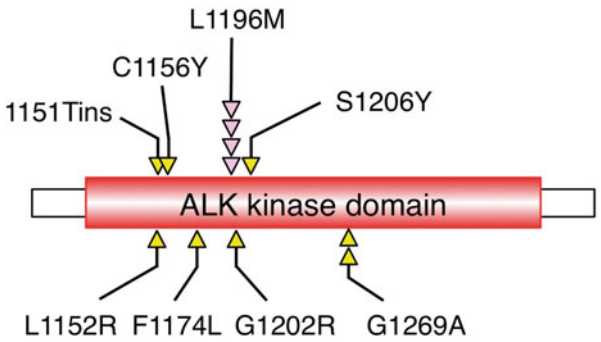

Fig. 18.2 Amino acid changes in the kinase domain of ALK responsible for tolerance to crizotinib. Various crizotinib resistance mutations have been identified in the kinase domain of ALK in *EML4-ALK*–positive tumours. *Arrowheads* indicate the numbers of patients identified for each mutation, with those for L1196M (gatekeeper mutation) being shown in *pink*

note, these two mutations arose not in the same cell clone, but separately in different cancer cells present in the same pleural effusion, reflecting a high mutation rate in the cancer genome.

Several additional mutations—including 1151Tins, L1152R, F1174L, G1202R, S1206Y, and G1269A—were separately shown to confer crizotinib resistance on cancer cells [20–23]. In total, ~30 % of drug tolerance cases can be explained by point mutations, insertions, or deletions in the kinase domain of EML4-ALK. The L1196 residue of ALK is the gatekeeper site, corresponding to T790 in EGFR and T315 in the PTK ABL, both of which are the most frequent resistance mutation sites for the targeted drugs, gefitinib and imatinib, respectively.

Other genetic alterations may also contribute to drug tolerance. Amplification of *EML4-ALK* has thus been observed in a small number of cases [22, 23]. Moreover, the EGFR signalling pathway has been found to be activated in some crizotinib-resistant tumours; in most of these instances, however, the up-regulation of this pathway is not due to mutational activation of EGFR [23], suggesting that increased production of ligands for EGFR family proteins or other mechanisms are responsible. *EGFR* or *KRAS* mutations have also been detected in crizotinib-resistant tumours [22], and amplification of *KIT* has been reported as a mechanism for drug tolerance [23].

18.2.5 Second-Generation ALK Inhibitors

Several second-generation ALK inhibitors—such as alectinib (Chugai), ceritinib (Novartis), AP26113 (Ariad), X-396 (Xcovery), PF-06463922 (Pfizer), CEP-37440 (Teva), RXDX-101 (Ignyta), TSR-011 (Tesaro) and ASP3026 (Astellas)—have been developed in an attempt to overcome the problem of crizotinib resistance. These compounds are more potent and more ALK-specific compared with crizotinib. Importantly, all of these agents are effective against *EML4-ALK*–positive tumours that harbour the crizotinib-resistant gatekeeper mutation L1196M. A phase 1/2 study of alectinib in crizotinib-naïve patients with *ALK* rearrangement–positive NSCLC yielded a remarkable response rate of 93.5 % [24]. On the other hand, the response rates for ceritinib and AP26113 in crizotinib-resistant patients were reported to be 73 % [25] and 67 % [26], respectively. Based on these data, ceritinib was approved as a therapeutic drug in U.S. as of April 2014, and alectinib was approved in Japan as of July 2014.

Variants of EML4-ALK harbouring point mutations that confer crizotinib resistance have been found to exhibit different sensitivities to second-generation ALK inhibitors. For instance, EML4-ALK(G1202R) is more sensitive to ASP3026 than to alectinib [23]. Rebiopsy (or serial biopsy) of tumour cells at the relapsed stage thus becomes important for selection of the most appropriate drug for a given patient.

18.3 *ROS1* Fusions

18.3.1 Types of ROS1 *Fusion Proteins*

Fusion of the ROS1 tyrosine kinase to SLC34A2 or CD74 in lung cancer was discovered by a phospho-proteomics approach in 2007 [10]. Subsequent analyses revealed many additional fusion partners for ROS1 including SDC4 [27], TPM3 [27], EZR [27], LRIG3 [27], CCDC6 [28], GOPC (also known as FIG) [29], and KDELR2 [30]. Among these various fusion proteins, CD74-ROS1 and SLC34A2-ROS1 appear to be the dominant types. The clinical characteristics of lung cancer positive for ROS1 fusion are similar to those that are positive for *ALK* rearrangements, with affected patients tending to have adenocarcinoma and an early disease onset as well as to be never- or light smokers.

18.3.2 Targeted Therapies

The NSCLC cell line HCC78 harbours the *SLC34A2-ROS1* fusion gene, the protein product of which is constitutively activated. ROS1 is as sensitive to crizotinib as is ALK, and treatment of HCC78 cells with crizotinib revealed that they are dependent on SLC34A2-ROS1 activity [31]. Furthermore, a patient with ROS1 fusion–positive NSCLC showed an almost complete response to treatment with crizotinib [31].

In addition to crizotinib, many other ALK inhibitors also effectively suppress ROS1 activity. Several compounds including crizotinib are thus in clinical trials for *ROS1* rearrangement–positive tumours. Mechanisms of drug tolerance in such tumours are also currently under investigation. A crizotinib resistance mutation (G2032R) in the kinase domain of CD74-ROS1 was identified in an individual with relapsed NSCLC positive for this fusion protein [32]. This mutation in CD74-ROS1 corresponds to the G1202R mutation in EML4-ALK (Fig. 18.2). Further examination is warranted to determine whether other crizotinib resistance mutations identified in EML4-ALK also arise in ROS1 fusions.

18.4 *RET* Fusions

18.4.1 KIF5B-RET *and Other* RET *Fusions*

With the use of different screening systems, four groups almost simultaneously identified *KIF5B-RET* as a fusion gene present in 1–2 % of lung adenocarcinoma cases [27, 33–35]. The two fusion partners both map to chromosome 10, with a small inversion involving the two loci giving rise to the gene fusion. Given that the KIF5B protein contains many dimerization motifs, various introns of *KIF5B* can

potentially serve as the site of fusion with *RET* and thereby generate an activated tyrosine kinase. In addition to *KIF5B*, other fusion partners of *RET* have been identified, albeit at lower frequencies, including *CCDC6* [27], *NCOA4* [36], and *TRIM33* [37]. Most fusion points in *RET* are located in intron 12, with the result that partner proteins become directly fused to the intracellular tyrosine kinase domain of RET. Similar to NSCLC positive for *ALK* or *ROS1* rearrangement, most *RET* fusion–positive tumours are adenocarcinomas, have an early onset, and develop in individuals who are never- or light smokers [27, 36]. In contrast to *ALK* rearrangement–positive tumours, however, tumour cells positive for *RET* fusions are poorly differentiated [36].

18.4.2 Targeted Therapies

Various RET inhibitors—including cabozantinib, vandetanib, and sunitinib—are under investigation for their therapeutic efficacy in individuals with NSCLC positive for *RET* fusions. An early study of cabozantinib published in 2013 demonstrated a partial response in two out of three patients [37]. Treatment of the *CCDC6-RET*–positive adenocarcinoma cell line LC-2/ad with vandetanib induced apoptosis associated with shutdown of phosphorylation (activation) of the target protein kinases AKT and ERK1/2 [38]. In contrast, knockdown of the fusion protein by RNA interference resulted in only partial suppression of the phosphorylation of these signalling molecules and did not prevent cell growth. Similarly, whereas the kinase inhibitor sorafenib blocked phosphorylation of the RET fusion protein, it failed to induce cell death. These results thus suggest that oncogenic proteins other than CCDC6-RET are activated in LC-2/ad cells, and that blockade of RET-mediated signalling alone may not be sufficient to exert antitumour activity. Whether this situation is LC-2/ad–specific or applicable to other *RET* fusion–positive tumours should be revealed by clinical trials of RET inhibitors.

18.5 Other Gene Fusions in Lung Cancer

18.5.1 FGFR *Family Fusions*

Fusions involving fibroblast growth factor receptor (FGFR) genes are unusual in that (1) in most instances, the tyrosine kinase gene is the 5′ partner, not the 3′ one (Fig. 18.3), and (2) they occur most frequently in squamous cell lung carcinoma. The fusion protein FGFR3-TACC3 was originally identified in glioblastoma, with the coiled-coil domains of TACC3 replacing the carboxyl terminus of FGFR3 [39], and it was subsequently found to be a recurrent genetic alteration in squamous cell carcinoma of the lung [11, 40]. One case positive for FGFR2-CCAR2 and another

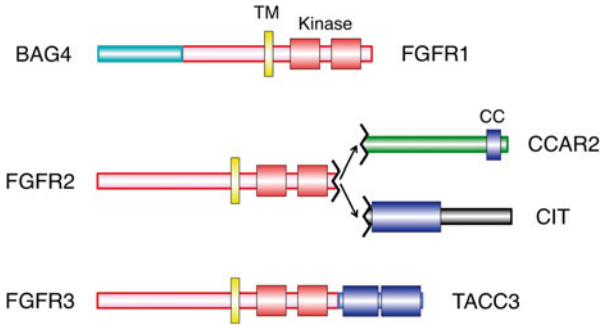

Fig. 18.3 Schematic structure of fusion proteins involving FGFR family members. Fusions of FGFR1, FGFR2, and FGFR3 are shown. *TM* transmembrane domain, *CC* coiled-coil domain

for BAG4-FGFR1 were discovered for the same disorder [40]. Another fusion of FGFR2, FGFR2-CIT, was identified in lung adenocarcinoma [28].

18.5.2 NTRK1 *Fusions*

Fusion of the neurotrophic tyrosine kinase receptor type 1 gene (*NTRK1*) to two different 5′ partners (*MPRIP* and *CD74*) was detected in female never-smokers with lung adenocarcinoma [41]. MPRIP also contains coiled-coil domains, with its fusion to the intracellular kinase domain of NTRK1 thus increasing kinase activity. Treatment of the *MPRIP-NTRK1*–positive patient with crizotinib (a weak inhibitor of NTRK1) resulted in successful disease control, albeit for a short period.

18.5.3 Other Fusions

Other PTK fusion genes identified in lung cancer include *AXL-MBIP* and *SCAF11-PDGFRA* [28]. Of note, the MBIP portion of the AXL-MBIP fusion protein contains leucine-zipper domains, which may function as a dimerization motif. Serine-threonine kinase genes have also been found to participate in gene fusions, such as *MAP4K3-PRKCE* [28], *BCAS3-MAP3K3* [28], *ERBB2IP-MAST4* [28], and *MARK4-ERCC2* [42].

Various nonkinase fusions have also been discovered, including KRAS-CDH13 [28], APLP2-TNFSF11 [28], ZFYVE9-CGA [28], TPD52L1-TRMT11 [28], RHPN2-PEPD [42], SIRT2-NPHS1 [42], NIPAL3-ATAD3B [42], UBFD1-CDH11 [42], GRHL2-PTPN12 [42], and GLE1-CCBL1 [42]. Whether or how these fusion proteins contribute to carcinogenesis remains to be determined.

18.6 Concluding Remarks

Chromosome rearrangements have been extensively searched for in lung cancer (as well as other cancer types) with the use of next-generation sequencers. Many of those identified are presumably "passenger mutations," merely reflecting chromosome instability in cancer cells. However, if a chromosome rearrangement results in the fusion of two genes in-frame, and if the fusion involves a protein kinase gene, then it warrants further investigation. *ALK* fusions and *ROS1* fusions, for instance, are essential growth drivers to which cancer cells become addicted and which are ideal targets for the development of therapeutic agents. Nevertheless, other types of fusion may also contribute to carcinogenesis, as exemplified by those involving transcription factor genes in leukaemia and prostate cancer.

As the number of targetable fusion proteins increases, it becomes a demanding task in the clinic to diagnose precisely which fusion event has taken place in a given tumour. Conventional diagnostics will be challenged in terms of both the time and budget required for such a determination. Novel strategies and techniques for diagnosis, such as those based on next-generation sequencers, are thus urgently needed. Given that a proto-oncogene can be activated by fusion to various partners in different tissues, as demonstrated by "ALKoma," [43] future diagnostics may override the tissue barrier and be uniformly applicable to all human cancers.

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D (2011) Global cancer statistics. CA Cancer J Clin 61:69–90
2. Mok TS, Wu YL, Thongprasert S, Yang CH, Chu DT, Saijo N, Sunpaweravong P, Han B, Margono B, Ichinose Y, Nishiwaki Y, Ohe Y, Yang JJ, Chewaskulyong B, Jiang H, Duffield EL, Watkins CL, Armour AA, Fukuoka M (2009) Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. N Engl J Med 361:947–957
3. Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S, Fujiwara S, Watanabe H, Kurashina K, Hatanaka H, Bando M, Ohno S, Ishikawa Y, Aburatani H, Niki T, Sohara Y, Sugiyama Y, Mano H (2007) Identification of the transforming *EML4-ALK* fusion gene in non-small-cell lung cancer. Nature 448:561–566
4. Soda M, Takada S, Takeuchi K, Choi YL, Enomoto M, Ueno T, Haruta H, Hamada T, Yamashita Y, Ishikawa Y, Sugiyama Y, Mano H (2008) A mouse model for *EML4-ALK*-positive lung cancer. Proc Natl Acad Sci U S A 105:19893–19897
5. Chen Z, Sasaki T, Tan X, Carretero J, Shimamura T, Li D, Xu C, Wang Y, Adelmant GO, Capelletti M, Lee HJ, Rodig SJ, Borgman C, Park SI, Kim HR, Padera R, Marto JA, Grey NS, Kung AL, Shapiro GI, Janne PA, Wong KK (2010) Inhibition of ALK, PI3K/MEK, and HSP90 in murine lung adenocarcinoma induced by EML4-ALK fusion oncogene. Cancer Res 70:9827–9836
6. Koivunen JP, Mermel C, Zejnullahu K, Murphy C, Lifshits E, Holmes AJ, Choi HG, Kim J, Chiang D, Thomas R, Lee J, Richards WG, Sugarbaker DJ, Ducko C, Lindeman N, Marcoux JP, Engelman JA, Grey NS, Lee C, Meyerson M, Janne PA (2008) EML4-ALK fusion gene and efficacy of an ALK kinase inhibitor in lung cancer. Clin Cancer Res 14:4275–4283

7. Takeuchi K, Choi YL, Soda M, Inamura K, Togashi Y, Hatano S, Enomoto M, Takada S, Yamashita Y, Satoh Y, Okumura S, Nakagawa K, Ishikawa Y, Mano H (2008) Multiplex reverse transcription-PCR screening for *EML4-ALK* fusion transcripts. Clin Cancer Res 14:6618–6624
8. Soda M, Isobe K, Inoue A, Maemondo M, Oizumi S, Fujita Y, Gemma A, Yamashita Y, Ueno T, Takeuchi K, Choi YL, Miyazawa H, Tanaka T, Hagiwara K, Mano H (2012) A prospective PCR-based screening for the *EML4-ALK* oncogene in non-small cell lung cancer. Clin Cancer Res 18:5682–5689
9. Takeuchi K, Choi YL, Togashi Y, Soda M, Hatano S, Inamura K, Takada S, Ueno T, Yamashita Y, Satoh Y, Okumura S, Nakagawa K, Ishikawa Y, Mano H (2009) KIF5B-ALK, a novel fusion oncokinase identified by an immunohistochemistry-based diagnostic system for ALK-positive lung cancer. Clin Cancer Res 15:3143–3149
10. Rikova K, Guo A, Zeng Q, Possemato A, Yu J, Haack H, Nardone J, Lee K, Reeves C, Li Y, Hu Y, Tan Z, Stokes M, Sullivan L, Mitchell J, Wetzel R, Macneill J, Ren JM, Yuan J, Bakalarski CE, Villen J, Kornhauser JM, Smith B, Li D, Zhou X, Gygi SP, Gu TL, Polakiewicz RD, Rush J, Comb MJ (2007) Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. Cell 131:1190–1203
11. Majewski IJ, Mittempergher L, Davidson NM, Bosma A, Willems SM, Horlings HM, de Rink I, Greger L, Hooijer GK, Peters D, Nederlof PM, Hofland I, de Jong J, Wesseling J, Kluin RJ, Brugman W, Kerkhoven R, Nieboer F, Roepman P, Broeks A, Muley TR, Jassem J, Niklinski J, van Zandwijk N, Brazma A, Oshlack A, van den Heuvel M, Bernards R (2013) Identification of recurrent FGFR3 fusion genes in lung cancer through kinome-centred RNA sequencing. J Pathol 230:270–276
12. Togashi Y, Soda M, Sakata S, Sugawara E, Hatano S, Asaka R, Nakajima T, Mano H, Takeuchi K (2012) KLC1-ALK: a novel fusion in lung cancer identified using a formalin-fixed paraffin-embedded tissue only. PLoS One 7:e31323
13. Fang DD, Zhang B, Gu Q, Lira M, Xu Q, Sun H, Qian M, Sheng W, Ozeck M, Wang Z, Zhang C, Chen X, Chen KX, Li J, Chen SH, Christensen J, Mao M, Chan CC (2014) HIP1-ALK, a novel ALK fusion variant that responds to crizotinib. J Thorac Oncol 9:285–294
14. Kwak EL, Bang YJ, Camidge DR, Shaw AT, Solomon B, Maki RG, Ou SH, Dezube BJ, Janne PA, Costa DB, Varella-Garcia M, Kim WH, Lynch TJ, Fidias P, Stubbs H, Engelman JA, Sequist LV, Tan W, Gandhi L, Mino-Kenudson M, Wei GC, Shreeve SM, Ratain MJ, Settleman J, Christensen JG, Haber DA, Wilner K, Salgia R, Shapiro GI, Clark JW, Iafrate AJ (2010) Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. N Engl J Med 363:1693–1703
15. Li T, Maus MK, Desai SJ, Beckett LA, Stephens C, Huang E, Hsiang J, Zeger G, Danenberg KD, Astrow SH, Gandara DR (2014) Large-scale screening and molecular characterization of EML4-ALK fusion variants in archival non-small-cell lung cancer tumor specimens using quantitative reverse transcription polymerase chain reaction assays. J Thorac Oncol 9:18–25
16. Yoshida A, Tsuta K, Nakamura H, Kohno T, Takahashi F, Asamura H, Sekine I, Fukayama M, Shibata T, Furuta K, Tsuda H (2011) Comprehensive histologic analysis of ALK-rearranged lung carcinomas. Am J Surg Pathol 35:1226–1234
17. Christensen JG, Zou HY, Arango ME, Li Q, Lee JH, McDonnell SR, Yamazaki S, Alton GR, Mroczkowski B, Los G (2007) Cytoreductive antitumor activity of PF-2341066, a novel inhibitor of anaplastic lymphoma kinase and c-Met, in experimental models of anaplastic large-cell lymphoma. Mol Cancer Ther 6:3314–3322
18. Shaw AT, Kim DW, Nakagawa K, Seto T, Crino L, Ahn MJ, De Pas T, Besse B, Solomon BJ, Blackhall F, Wu YL, Thomas M, O'Byrne KJ, Moro-Sibilot D, Camidge DR, Mok T, Hirsh V, Riely GJ, Iyer S, Tassell V, Polli A, Wilner KD, Janne PA (2013) Crizotinib versus chemotherapy in advanced ALK-positive lung cancer. N Engl J Med 368:2385–2394
19. Choi YL, Soda M, Yamashita Y, Ueno T, Takashima J, Nakajima T, Yatabe Y, Takeuchi K, Hamada T, Haruta H, Ishikawa Y, Kimura H, Mitsudomi T, Tanio Y, Mano H (2010) EML4-

ALK mutations in lung cancer that confer resistance to ALK inhibitors. N Engl J Med 363:1734–1739
20. Sasaki T, Okuda K, Zheng W, Butrynski J, Capelletti M, Wang L, Grey NS, Wilner K, Christensen JG, Demetri G, Shapiro GI, Rodig SJ, Eck MJ, Janne PA (2010) The neuroblastoma-associated F1174L ALK mutation causes resistance to an ALK kinase inhibitor in ALK-translocated cancers. Cancer Res 70:10038–10043
21. Sasaki T, Koivunen J, Ogino A, Yanagita M, Nikiforow S, Zheng W, Lathan C, Marcoux JP, Du J, Okuda K, Capelletti M, Shimamura T, Ercan D, Stumpfova M, Xiao Y, Weremowicz S, Butaney M, Heon S, Wilner K, Christensen JG, Eck MJ, Wong KK, Lindeman N, Grey NS, Rodig SJ, Janne PA (2011) A novel ALK secondary mutation and EGFR signaling cause resistance to ALK kinase inhibitors. Cancer Res 71:6051–6060
22. Doebele RC, Pilling AB, Aisner DL, Kutateladze TG, Le AT, Weickhardt AJ, Kondo KL, Linderman DJ, Heasley LE, Franklin WA, Varella-Garcia M, Camidge DR (2012) Mechanisms of resistance to crizotinib in patients with ALK gene rearranged non-small cell lung cancer. Clin Cancer Res 18:1472–1482
23. Katayama R, Shaw AT, Khan TM, Mino-Kenudson M, Solomon BJ, Halmos B, Jessop NA, Wain JC, Yeo AT, Benes C, Drew L, Saeh JC, Crosby K, Sequist LV, Iafrate AJ, Engelman JA (2012) Mechanisms of acquired crizotinib resistance in ALK-rearranged lung cancers. Sci Transl Med 4:120ra117
24. Seto T, Kiura K, Nishio M, Nakagawa K, Maemondo M, Inoue A, Hida T, Yamamoto N, Yoshioka H, Harada M, Ohe Y, Nogami N, Takeuchi K, Shimada T, Tanaka T, Tamura T (2013) CH5424802 (RO5424802) for patients with ALK-rearranged advanced non-small-cell lung cancer (AF-001JP study): a single-arm, open-label, phase 1–2 study. Lancet Oncol 14:590–598
25. Shaw AT, Mehra R, Kim D-W, Felip E, Quan Man Chow L, Camidge DR, Tan DS-W, Vasteenkiste JF, Sharma S, De Pas T, Wolf J, Katayama R, Lau Y-YY, Goldwasser M, Boral A, Engelman JA (2013) Clinical activity of the ALK inhibitor LDK378 in advanced, ALK-positive NSCLC. J Clin Oncol 31(suppl):8010
26. Camidge DR, Bazhenova L, Salgia R, Weiss GJ, Langer CJ, Shaw AT, Narasimhan NI, Dorer DJ, Rivera VM, Zhang J, Clackson T, Haluska FG, Gettinger SN (2013) First-in-human dose-finding study of the ALK/EGFR inhibitor AP26113 in patients with advanced malignancies: updated results. J Clin Oncol 31(suppl):8031
27. Takeuchi K, Soda M, Togashi Y, Suzuki R, Sakata S, Hatano S, Asaka R, Hamanaka W, Ninomiya H, Uehara H, Choi YL, Satoh Y, Okumura S, Nakagawa K, Mano H, Ishikawa Y (2012) RET, ROS1 and ALK fusions in lung cancer. Nat Med 18:378–381
28. Seo JS, Ju YS, Lee WC, Shin JY, Lee JK, Bleazard T, Lee J, Jung YJ, Kim JO, Shin JY, Yu SB, Kim J, Lee ER, Kang CH, Park IK, Rhee H, Lee SH, Kim JI, Kang JH, Kim YT (2012) The transcriptional landscape and mutational profile of lung adenocarcinoma. Genome Res 22:2109–2119
29. Suehara Y, Arcila M, Wang L, Hasanovic A, Ang D, Ito T, Kimura Y, Drilon A, Guha U, Rusch V, Kris MG, Zakowski MF, Rizvi N, Khanin R, Ladanyi M (2012) Identification of KIF5B-RET and GOPC-ROS1 fusions in lung adenocarcinomas through a comprehensive mRNA-based screen for tyrosine kinase fusions. Clin Cancer Res 18:6599–6608
30. Govindan R, Ding L, Griffith M, Subramanian J, Dees ND, Kanchi KL, Maher CA, Fulton R, Fulton L, Wallis J, Chen K, Walker J, McDonald S, Bose R, Ornitz D, Xiong D, You M, Dooling DJ, Watson M, Mardis ER, Wilson RK (2012) Genomic landscape of non-small cell lung cancer in smokers and never-smokers. Cell 150:1121–1134
31. Bergethon K, Shaw AT, Ignatius Ou SH, Katayama R, Lovly CM, McDonald NT, Massion PP, Siwak-Tapp C, Gonzalez A, Fang R, Mark EJ, Batten JM, Chen H, Wilner KD, Kwak EL, Clark JW, Carbone DP, Ji H, Engelman JA, Mino-Kenudson M, Pao W, Iafrate AJ (2012) ROS1 rearrangements define a unique molecular class of lung cancers. J Clin Oncol 30:863–870

32. Awad MM, Katayama R, McTigue M, Liu W, Deng YL, Brooun A, Friboulet L, Huang D, Falk MD, Timofeevski S, Wilner KD, Lockerman EL, Khan TM, Mahmood S, Gainor JF, Digumarthy SR, Stone JR, Mino-Kenudson M, Christensen JG, Iafrate AJ, Engelman JA, Shaw AT (2013) Acquired resistance to crizotinib from a mutation in CD74-ROS1. N Engl J Med 368:2395–2401
33. Lipson D, Capelletti M, Yelensky R, Otto G, Parker A, Jarosz M, Curran JA, Balasubramanian S, Bloom T, Brennan KW, Donahue A, Downing SR, Frampton GM, Garcia L, Juhn F, Mitchell KC, White E, White J, Zwirko Z, Peretz T, Nechushtan H, Soussan-Gutman L, Kim J, Sasaki H, Kim HR, Park SI, Ercan D, Sheehan CE, Ross JS, Cronin MT, Janne PA, Stephens PJ (2012) Identification of new ALK and RET gene fusions from colorectal and lung cancer biopsies. Nat Med 18:382–384
34. Kohno T, Ichikawa H, Totoki Y, Yasuda K, Hiramoto M, Nammo T, Sakamoto H, Tsuta K, Furuta K, Shimada Y, Iwakawa R, Ogiwara H, Oike T, Enari M, Schetter AJ, Okayama H, Haugen A, Skaug V, Chiku S, Yamanaka I, Arai Y, Watanabe S, Sekine I, Ogawa S, Harris CC, Tsuda H, Yoshida T, Yokota J, Shibata T (2012) KIF5B-RET fusions in lung adenocarcinoma. Nat Med 18:375–377
35. Ju YS, Lee WC, Shin JY, Lee S, Bleazard T, Won JK, Kim YT, Kim JI, Kang JH, Seo JS (2012) A transforming KIF5B and RET gene fusion in lung adenocarcinoma revealed from whole-genome and transcriptome sequencing. Genome Res 22:436–445
36. Wang R, Hu H, Pan Y, Li Y, Ye T, Li C, Luo X, Wang L, Li H, Zhang Y, Li F, Lu Y, Lu Q, Xu J, Garfield D, Shen L, Ji H, Pao W, Sun Y, Chen H (2012) RET fusions define a unique molecular and clinicopathologic subtype of non-small-cell lung cancer. J Clin Oncol 30:4352–4359
37. Drilon A, Wang L, Hasanovic A, Suehara Y, Lipson D, Stephens P, Ross J, Miller V, Ginsberg M, Zakowski MF, Kris MG, Ladanyi M, Rizvi N (2013) Response to cabozantinib in patients with RET fusion-positive lung adenocarcinomas. Cancer Discov 3:630–635
38. Suzuki M, Makinoshima H, Matsumoto S, Suzuki A, Mimaki S, Matsushima K, Yoh K, Goto K, Suzuki Y, Ishii G, Ochiai A, Tsuta K, Shibata T, Kohno T, Esumi H, Tsuchihara K (2013) Identification of a lung adenocarcinoma cell line with CCDC6-RET fusion gene and the effect of RET inhibitors in vitro and in vivo. Cancer Sci 104:896–903
39. Singh D, Chan JM, Zoppoli P, Niola F, Sullivan R, Castano A, Liu EM, Reichel J, Porrati P, Pellegatta S, Qiu K, Gao Z, Ceccarelli M, Riccardi R, Brat DJ, Guha A, Aldape K, Golfinos JG, Zagzag D, Mikkelsen T, Finocchiaro G, Lasorella A, Rabadan R, Iavarone A (2012) Transforming fusions of FGFR and TACC genes in human glioblastoma. Science 337:1231–1235
40. Wu YM, Su F, Kalyana-Sundaram S, Khazanov N, Ateeq B, Cao X, Lonigro RJ, Vats P, Wang R, Lin SF, Cheng AJ, Kunju LP, Siddiqui J, Tomlins SA, Wyngaard P, Sadis S, Roychowdhury S, Hussain MH, Feng FY, Zalupski MM, Talpaz M, Pienta KJ, Rhodes DR, Robinson DR, Chinnaiyan AM (2013) Identification of targetable FGFR gene fusions in diverse cancers. Cancer Discov 3:636–647
41. Vaishnavi A, Capelletti M, Le AT, Kako S, Butaney M, Ercan D, Mahale S, Davies KD, Aisner DL, Pilling AB, Berge EM, Kim J, Sasaki H, Park SI, Kryukov G, Garraway LA, Hammerman PS, Haas J, Andrews SW, Lipson D, Stephens PJ, Miller VA, Varella-Garcia M, Janne PA, Doebele RC (2013) Oncogenic and drug-sensitive NTRK1 rearrangements in lung cancer. Nat Med 19:1469–1472
42. Kim SC, Jung Y, Park J, Cho S, Seo C, Kim J, Kim P, Park J, Seo J, Kim J, Park S, Jang I, Kim N, Yang JO, Lee B, Rho K, Jung Y, Keum J, Lee J, Han J, Kang S, Bae S, Choi SJ, Kim S, Lee JE, Kim W, Kim J, Lee S (2013) A high-dimensional, deep-sequencing study of lung adenocarcinoma in female never-smokers. PLoS One 8:e55596
43. Mano H (2012) ALKoma: a cancer subtype with a shared target. Cancer Discov 2:495–502

Chapter 19
Colon and Ovarian Translocations

Paul T. Spellman

Contents

19.1 Introduction 418
19.2 Structural Rearrangements 419
19.3 Specific Fusions in Ovarian Cancer 420
19.4 Specific Fusions in Colon Cancer 422
19.5 Functions of Rearrangements in Colon Cancer Genomes 423
19.6 Conclusion 425
Acknowledgements 425
References 425

Abstract Two of the first types of cancer genomes analysed, ovarian and colorectal, show substantial differences in many key aspects (*e.g.* copy number profiles and number of point mutant driving lesions) but both now have evidence for structural variants that drive these tumours. About ten papers have published one or more recurrent drivers in these tumour types with a few important findings. First, there is a wide range in the number of structural rearrangements from just a few to hundreds per cancer genome. Second, most common rearrangements mirror the patterns seen in other tumour types where the exact gene pair is less important than the types of genes that are being rearranged. Finally, these tumour types emphasize the complexity of the problem from technical as well as epidemiological perspectives – the validation of rearrangements as being recurrent (and thus as likely drivers) remains a major challenge.

Keywords Ovarian • Colon • Cancer • *TCF7L2* • *ESRRA*

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19.1 Introduction

High-grade serous ovarian cancer (ovarian cancer) and adenocarcinoma of the colon and rectum (colon cancer) are an interesting pair of cancer types to compare and contrast in considering structural rearrangements that drive oncogenesis. From a clinical perspective, these cancers are quite distinct. Ovarian cancer is nearly always identified late in progression with widely disseminated disease throughout the abdomen and is lethal in the vast majority of cases. Colon cancer, on the other hand, is often identified at an early stage; even as precancerous lesions. Although late stage cancer has poor outcomes, the majority of individuals diagnosed with colon cancer survive.

These two tumours were amongst the first subjected to systematic genomic interrogation involving high-resolution copy number analysis and exome sequencing for hundreds of distinct tumours for each type, as well as a more limited whole genome sequencing analysis [1]. Further analysis, particularly as part of the "Pan-Cancer" efforts [2] has highlighted specific commonalities and differences between the first 12 tumour types, examined by The Cancer Genome Atlas (TCGA). Additionally, both tumour types have been the focus of more targeted analyses to specifically identify structural rearrangements, usually through targeted RNAseq to identify fusion transcripts that are indicative of underlying genomic rearrangements.

A first analysis of structural rearrangements can start with an examination of DNA copy number, which might inform us as to the level and prevalence of rearrangements in the cancer genome. As can be seen in Fig. 19.1, ovarian cancer has massive numbers of copy number changes while the copy number patterns of colon cancer are far more muted. Figure 19.1 emphasizes the large scale copy number gains and losses which account for the majority of altered DNA content but the minority of all chromosome breakpoints.

Work preceding TCGA analysed the copy number spectrum of these two diseases, finding that there are approximately 30 focal copy number changes per cancer genome (focal is defined as smaller than a chromosome; median is 1.8 megabases) in colon cancer and approximately 50 copy number changes per genome in ovarian cancer [3]. The balance between focal gains and losses is quite closely matched in both cases. About 20 % of the colon cancer copy number events are large (i.e. on the order of a chromosome arm), while 10 % of the ovarian copy number events are arm-level events [3]. This result implies there must be a very large number of rearrangements in each cancer genome – the amount of focal copy number changes is a lower bound on rearrangements because it cannot identify copy neutral rearrangements nor can it identify very small rearrangements. Analysing the public copy number data using the GISTIC analysis from the Broad GDAC (http://gdac.broadinstitute.org) finds that there are 135 copy number breakpoints in colon cancer and 360 copy number breakpoints in ovarian cancer, which implies that there a very large number of potential functional rearrangements in these cancer genomes.

A comparison of the number of significantly mutated genes inferred to be oncogenic drivers shows far more drivers in colon cancer (32) than in ovarian cancer (6) and the prevalence of such drivers is far higher; 4.2 per tumour in colon cancers

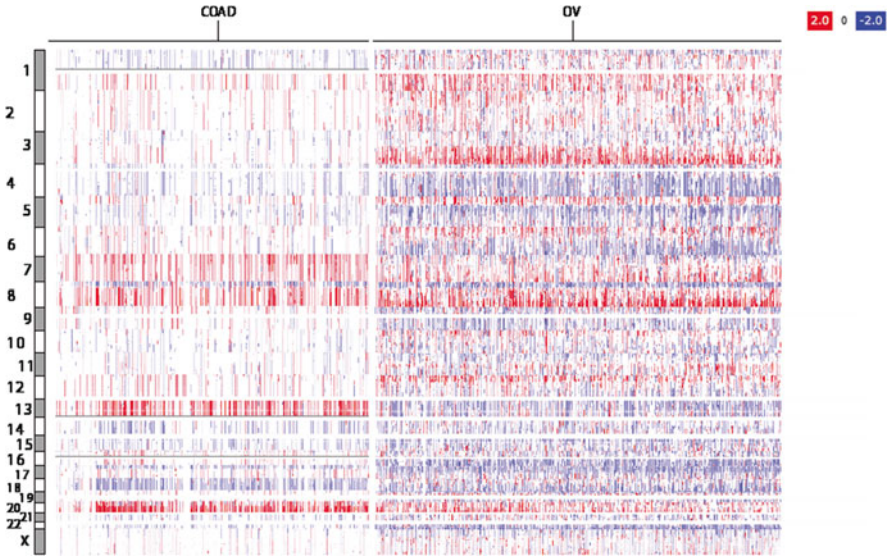

Fig. 19.1 Copy number data for TCGA ovarian serous cystadenocarcinoma (OV) and TCGA colon adenocarcinoma (COAD). 569 OV and 427 COAD samples from Affymetrix SNP6 platform are shown with blue indicating loss and red indicating gain. The pattern of copy number change in colon is quite simple, with most chromosomes showing normal copy number, while the pattern for ovarian is much more complicated, with nearly all chromosomes subject to copy number changes. Copy number changes were analysed at Broad using Firehose GISTIC 21 pipeline. Publicly available Level 4 GISTIC 2 data from Firehose (http://gdac.broadinstitute.org/runs/analyses__2014_04_16/data/COAD/20140416/) was used for generating the copy number heatmap (Figure reprinted by permission from Macmillan Publishers Limited: Nature, Cancer Genome Atlas Network [18])

versus 0.96 per tumour in ovarian cancers, as determined by MutSig2.0 [4] from the July 15, 2014 Broad GDAC Firehose run [5]. This is important because it should flavour our interpretation of the types of structural rearrangements that might be present in a cancer genome, and it might also influence their overall frequency. Indeed, the Pan-Can analysis of 12 tumour types puts ovarian and colon cancer on opposite ends of a spectrum comparing mutations and copy number drivers, colon is "M-class" (primarily driven by recurrent point mutations) while ovarian is "C-class" (primarily driven by recurrent copy number changes) [6]. These analyses suggest structural rearrangements that create fusion genes might be more common, or more important, in ovarian cancer than in colon cancer.

19.2 Structural Rearrangements

Genomic rearrangements are now being systematically identified in whole genome studies (see Chaps. 2 and 5). It is readily apparent there is substantial variation in the number of rearrangements in a cancer genome, with fewer than 10 in some genomes

and on the order of 1,000 in other genomes [7]. The cause of these rearrangements can either be deletions, tandem insertions or translocations [7]. One of the most exciting findings has been that structural rearrangements often involve complex rearrangements with many chromosomes involved [8, 9]. In ovarian cancer there is evidence for a tandem duplication phenotype [10].

19.3 Specific Fusions in Ovarian Cancer

I start the discussion of specific structural rearrangements with ovarian cancer. There are only three examples of recurrent rearrangements in this tumour type and they are all recent, having been published in just the past few years.

The first report of a recurrent fusion transcript in ovarian cancer was of the *ESRRA* gene to *TEX40/TEX40* [11]. The structure of the fusion is shown in Fig. 19.2.

Identification of *ESRRA-TEX40* was through a fairly typical fusion gene pipeline, analysing RNAseq data from one dozen cases for evidence of discordant gene mappings. Of some concern, the initial screening was done in pooled samples, which were later tested for evidence of fusions. An appropriate control was that the *ESRRA-TEX40* fusion was tested in a substantial number of ovarian cancers from distinct research sites (Fred Hutchinson Cancer Research Center, FHCRC and British Colombia Cancer Agency BCCA), and while its presence was lower in that population, it was independently identified. The preliminary estimate was 7–26 % of cases were positive for this fusion.

"Nine of the 42 cases screened at the FHCRC and 1 of the 25 cases screened at the BCCA were fusion-positive" [11].

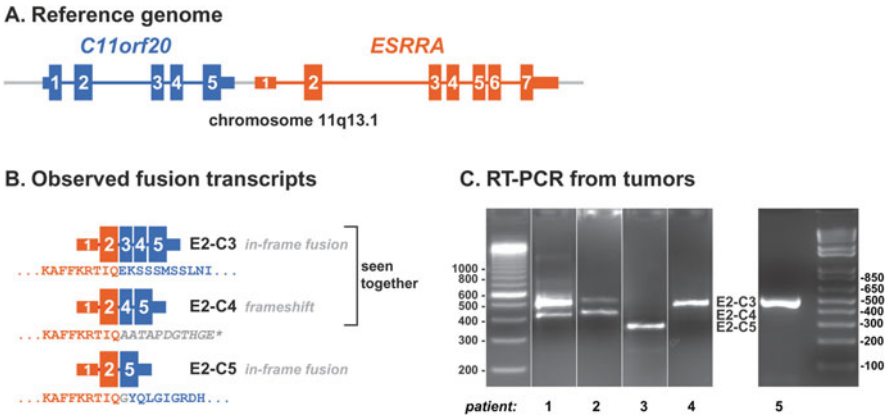

Fig. 19.2 (From Salzman 2011). Fusion transcript of *TEX40/c11orf20* to *ESRRA*. (**a**) The genomic structure of the *TEX40* and *ESSRA* genes. (**b**) Observed fusion transcripts. (**c**) RT-PCR from individual tumours showing presence of the fusion transcripts (Figure reprinted by permission from Macmillan Publishers Limited: Science-Business eXchange. Salzman et al. [11])

RNAseq detects a fusion transcript that merges exon 2 of ESRRA to either exon 3, 4, or 5 of TEX40. Given the biology of ovarian cancer (an oestrogen-driven tumour type), it is suggestive that a close homolog of the oestrogen receptor gene (*ESR1*) should be involved. No mechanistic study of the fusion has yet been published but the ESRRA-TEX40 story does not end with the first Salzman effort. A negative report could not find evidence for the fusion in RNAseq data from 10 ovarian cancers, and no evidence of the fusion could be found in PCR of reverse transcribed cDNA from 230 ovarian cancer cases [12]. Micci et al. suggest the fact the genes are only 11,000 basepairs apart from one another is somehow responsible for artificially observing the fusion transcript. Allowed to comment, Salzman and colleagues reported RNAseq from 420 cases in the TCGA ovarian cancer dataset [13] identified three examples of the *ESRRA-TEX40* fusion. Recalculating the frequency of the fusion based on the full set of data it was inferred that while recurrent, the fusion has a frequency on the order of 1 % of ovarian cancer cases (Salzman et al. 2014, http://www.plosbiology.org/annotation/listThread.action?root=78349. Response to reference: [12]). It seems unlikely that the final chapter has been written on *ESRRA-TEX40*.

The recurrent copy number aberrations in ovarian cancer have long suggested some of them might lead to recurrent rearrangements that could not be easily identified in conventional karyotyping. Following up on this idea, it was found 11q13.2 is routinely joined to 19p13.2 using spectral karyotyping (SKY) [14], and in the SKOV-3 ovarian cancer cell line, this breakpoint joins the *HOOK2* gene to *SCTN3* [15]. Expanding this observation into clinical specimens, fluorescence in situ hybridization (FISH) was used to identify tumours that co-localized these two chromosome bands [16]. The rearrangement was found in 45 % of all cases they analysed but it was highly biased toward low/intermediate-grade tumours, not those that we would call high-grade serous ovarian cancers [16]. Further, the boundaries of the rearrangement are not clear, meaning that *HOOK2* and *SCTN3* are not always at the junctions of the rearrangements.

The final effort to identify fusion transcripts created by rearrangements of the ovarian cancer genome identified 356 fusion transcript candidates [17]. The group used a similar chimaeric read approach to the Salzman effort; generating RNAseq data from seven cases. The group found that 16 candidates had been previously identified. The 47 best candidate fusions were selected and 15 of the fusions were validated using PCR from cDNA. Six of the fusions are interchromosomal, or long-range intrachromosomal events. The other nine fusions involved co-located gene pairs in the genome and are likely caused by transcriptional read-through events from one gene into the adjacent gene. The six events considered likely to be caused by rearrangements in the genome were further validated by RT-PCR in RNA from 28 additional cancer cases, 10 normal ovarian samples, and 4 normal fallopian tube samples. Of the six, just two were restricted to the cancers and four of the six involved chromosome 19.

Of the two fusions reported in Kannan et al. [17], one fusing *CDKN2D* to *WDFY2* was recurrent in 20 % of cases (12 of 60). The fusion is caused by a chromosomal rearrangement of chromosome 19 at about 10.6 megabases to chromosome 13 at

about 52.2 megabases. Mapping of the breakpoints by long-range PCR in one case allowed it to be determined the rearrangement boundaries were not universally the same. The protein encoded by the fusion transcript is likely a shortened form of the WDFY2 protein. The fusion transcript is expressed in the ovarian cancer cell line OV-90, which suggests an easy approach to study its mechanism.

Obviously the community has not fully vetted the frequency or impact of the rearrangements that have been found, with disagreements as to their frequency and meaning. What does seem evident is there are unlikely to be a large number of highly frequent aberrations, and the existence of even a small number of frequent (say >20 %) rearrangements is in doubt. A distinct possibility is there will be a very large number of rare but important drivers, some of which will fall into the class of fusions like ESRRA-TEX40, where some reasonable understanding of the biology is present. Others will look like 11q13.2-19p13.2, where it is not clear any gene fusions are important.

19.4 Specific Fusions in Colon Cancer

In comparison to ovarian cancer, there is a larger community working on recurrent rearrangement detection in colon cancer, and the community is having better success verifying rearrangements detected by other groups. In total, there are at least three recurrent gene fusions and the implication of many more functional, but perhaps more rare, fusions.

The first effort to identify rearrangements in colon cancer focused on the use of whole genomes sequencing from nine colon cancers [9]. As was seen in the broader analysis [7], there was a wide range of rearrangements detected in each colon tumour, with as few as 5 and as many as 182, for an average of 75 rearrangements each. As these rearrangements were detected from ~30× whole genome sequence, exact breakpoints could be found, making validation of the rearrangements considerably easier. In total, 92 % of all predicted rearrangements were validated by PCR from genome DNA. Further, there was ample evidence of complex rearrangements involving many chromosomes (Fig. 19.3), suggesting issues of tumour contamination by normal or perhaps, the mechanisms encouraging point mutations are the same as the mechanisms facilitating rearrangements. There was a positive correlation between the number of somatic substitutions and the number of somatic rearrangements.

Analysing the specific mutations Bass et al. [9] found, 82 % of the rearrangements are intrachromosomal (just 18 % are interchromosomal), but of the intrachromosomal events, about half were long-range (defined as chromosomal regions greater than 1 Mb apart). Despite the very large number of rearrangements (>600), just 11 were found to cause rearrangements predicted to encode for in-frame fusion proteins. The group then screened each of these rearrangements in cDNA from 97 tumours and found one, a fusion of *VTI1A* to *TCF7L2*, in 3 of the 97 tumours. Function of the TCF7L2 fusion is discussed in more detail below.

Also using whole genome sequence approaches, TCGA published an analysis of 97 whole genomes sequenced to approximately 10× clone coverage [18]. The decrease

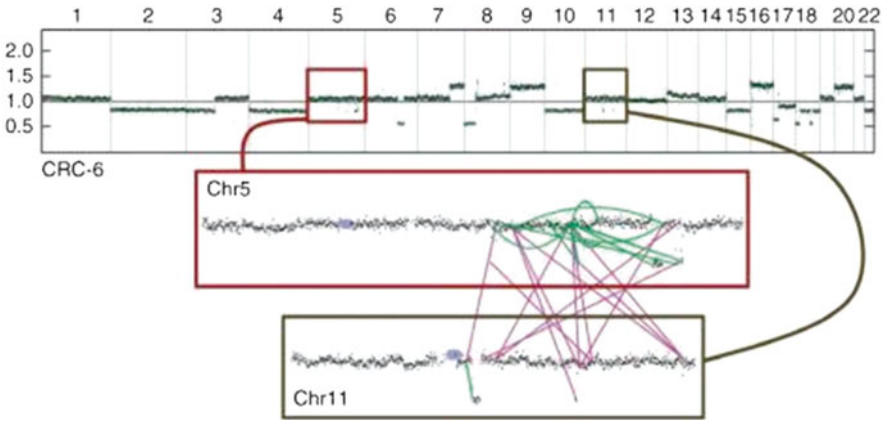

Fig. 19.3 Complex structural rearrangements in the genome of colon cancer from Bass et al. [9]. Chromosomes are labelled about the copy number plot at the top of the image. A very large number of rearrangements are found between chromosomes 5 and 11 spread across a large section of each chromosomes (lower two panels, *red lines* linking rearrangements) (Figure reprinted by permission from Macmillan Publishers Limited: Nature Genetics, Bass et al. [9] doi: 10.1101/gr.4247306)

in sequencing depth is important to recognize – it greatly diminished the power of the rearrangement detection. In these 97 whole genomes, just 250 rearrangements were detected (range 0–10, mean 2.6). On average, the 3× decrease in sequencing depth decreased the power by 30-fold (assuming the true means were equivalent). Somewhat surprisingly, the rate of predicted in-frame fusions was much higher, with 18 detected (18/250 vs 11/620). Of these 18 in-frame fusions, 3 were predicted to fuse *NAV2* to *TCF7L1* (located on chromosomes 11 and 2 respectively). Additionally, the TCGA report highlights the potential role of rearrangements in gene inactivation events, 21 cases had rearrangements expected to inactivate the *TTC28* gene.

The final major study in colon cancer utilized RNAseq to identify fusions. The Largaespada group sequenced RNA from 68 colon cancers and found 36 recurrent rearrangements [19]. Of those rearrangements, a few were characterized in more depth because they were predicted to alter Wnt signalling by altering R-spondin regulation. Specifically, *RSPO2* was involved in rearrangement in 2 of 68 cases, whereas *RSPO3* was involved in rearrangements of 5 of the 6 cases.

19.5 Functions of Rearrangements in Colon Cancer Genomes

The studies from Bass et al. [9] and from Seshagiri et al. [19] both characterized a few pathways biologically and highlighted how some of these aberrations are likely to function. The Bass work indicated TCF7L2 was important (and the TCGA work

identified its close homolog TCF7L1), whereas the Largaespada effort identified the R-spondins, RSPO2 and RSPO3.

TCF7L2 encodes the TCF4 transcription factor, which dimerizes with CTNNB1 in to regulate the genes responsible for intestinal epithelial cell differentiation and proliferation. As mentioned, the fusions involving both *TCF7L1* and *TCF7L2* are predicted to make in-frame fusions that include nearly the entire coding sequence of *TCF7L1* or *TCF7L2* [9, 18]. In all likelihood, these fusions are functional. The fusion of *VTI1A* to *TCF7L2* is caused by an intrachromosomal deletion, and in the NCI-H508 cell line, this deletion is 540,000 basepairs [9]. Induction of RNA interference-mediated knockdown of *VTI1A-TCF7L2* in NCI-H508 resulted in substantial abatement of anchorage independent-growth, strongly implying its oncogenic activity (Bass 2011).

Following the genomic characterization work, a newer study has added confusion to our understanding of the fusion transcript *VTI1A-TCF7L2*. Nome et al. [20] found a high rate of the fusion transcript in cancer tissue but also a high rate of the fusion transcript in normal colon tissues and other normal tissues. Further, the group found a frequent chimaeric transcript involving the RP11-57H14.3 gene with TCF7L2 (Fig. 19.4), [20], adding even more confusion to our understanding of the locus. The RP11-57H14.3 to TCF7L2 fusion is in non-canonical gene order, suggesting either a missed genomic rearrangement or a circular RNA [21]. It seems highly likely TCF7L2 is a functionally important molecule in the biology of colon cancer, but there is clearly much more work to understand its regulation – both in normal tissues and in cancer.

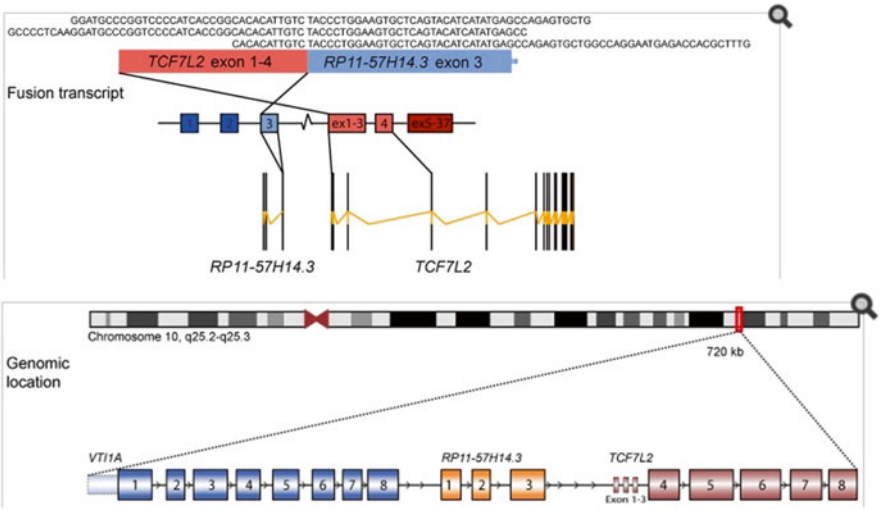

Fig. 19.4 TCF7L2 transcript fusions in colon cancer from Nome et al. [20]. (*top*) Sequence reads that support the fusion transcript linking exons 1–4 of *TCF7L2* to exon 3 of RP11-57H14.3. (*bottom*) The genomic region encoding *VTI1A*, RP11-57H14.3 and *TCF7L2* (Figure reprinted from Nome et al. [20])

The R-spondins have very plausible biology associated with colon cancer as they are responsible for activation of Wnt signalling, which has long been known to regulate colon cancer biology [22]. The *RSPO* fusions have been analysed in some detail. In the initial publication [19], a fusion of *EIF3E* to *RSPO2* caused by a 100,000 basepair deletion and a *PTPRK-RSPO3* fusion (both on chromosome 6q22.2-q22.3) were observed. The *RSPO3* fusion transcript is predicted to encode full-length protein. Functional validation of the fusion transcripts indicated that they retained function [19]. A follow-up study by another group found the *EIF3E-RSPO2* fusion in 2 of 75 cases and the *PTPRK-RSPO3* fusion in 1 of 75 cases [23].

19.6 Conclusion

Summarizing the biology of the rearrangements in ovarian and colon cancer leads us to a fuzzy picture. Structural rearrangements in the genome of ovarian and colon cancers are clearly an important component of most, if not all, of the oncogenic lesions that cause cancer. Nonetheless, the field is clearly in its infancy, and it is not clear if oncogenic rearrangements will be found in just a small fraction of cases or in most cases. The evidence to date is admittedly confusing, with high profile and exciting observations fading somewhat when deeply scrutinized. The examples identified to date often mirror the biology of the common and more prevalent rearrangements. Intrachromosomal rearrangements (either deletions or rearrangements) are most common, as in the case of *TMPRSS2-ERG* in prostate cancer [24], while interchromosomal rearrangements are less common but likely important. Some hope the systematic sequencing projects, like TCGA and ICGC, would have firmly answered such questions, and perhaps they will; but arguably, the sensitivity and specificity of these large projects is not quite where it needs to be to conclude that structural rearrangements driving cancer are truly rare. It seems likely, as the technology continues to improve, the true answer will become clear.

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References

1. Cancer Genome Atlas Research Network (2011) Integrated genomic analyses of ovarian carcinoma. Nature 474:609–615. doi:10.1038/nature10166
2. The Cancer Genome Atlas Research Network, Weinstein JN, Collisson EA, Mills GB, Shaw KRM et al (2013) The cancer genome atlas Pan-cancer analysis project. Nat Genet 45:1113–1120. doi:10.1038/ng.2764
3. Beroukhim R, Mermel CH, Porter D, Wei G, Raychaudhuri S et al (2010) The landscape of somatic copy-number alteration across human cancers. Nature 463:899–905. doi:10.1038/nature08822

4. Broad Institute TCGA Genome Data Analysis Center (2013) Breast invasive carcinoma (Primary solid tumor cohort) – 21 April 2013: mutation analysis (MutSig v2.0). Available: http://dx.doi.org/10.7908/C1JS9NC5. Accessed 26 Sept 2014
5. Broad GDAC Firehose (n.d.). Available: http://gdac.broadinstitute.org/. Accessed 26 Sept 2014
6. Ciriello G, Miller ML, Aksoy BA, Senbabaoglu Y, Schultz N et al (2013) Emerging landscape of oncogenic signatures across human cancers. Nat Genet 45:1127–1133. doi:10.1038/ng.2762
7. Yang L, Luquette LJ, Gehlenborg N, Xi R, Haseley PS et al (2013) Diverse mechanisms of somatic structural variations in human cancer genomes. Cell 153:919–929. doi:10.1016/j.cell.2013.04.010
8. Volik S, Raphael BJ, Huang G, Stratton MR, Bignel G et al (2006) Decoding the fine-scale structure of a breast cancer genome and transcriptome. Genome Res 16:394–404. doi:10.1101/gr.4247306
9. Bass AJ, Lawrence MS, Brace LE, Ramos AH, Drier Y et al (2011) Genomic sequencing of colorectal adenocarcinomas identifies a recurrent VTI1A-TCF7L2 fusion. Nat Genet 43:964–968. doi:10.1038/ng.936
10. McBride DJ, Etemadmoghadam D, Cooke SL, Alsop K, George J et al (2012) Tandem duplication of chromosomal segments is common in ovarian and breast cancer genomes. J Pathol 227:446–455. doi:10.1002/path.4042
11. Salzman J, Marinelli RJ, Wang PL, Green AE, Nielsen JS, et al (2011) ESRRA-C11orf20 is a recurrent gene fusion in serous ovarian carcinoma. PLoS Biol 9. doi:10.1371/journal.pbio.1001156
12. Micci F, Panagopoulos I, Thorsen J, Davidson B, Tropé CG et al (2014) Low frequency of ESRRA–C11orf20 fusion gene in ovarian carcinomas. PLoS Biol 12:e1001784. doi:10.1371/journal.pbio.1001784
13. Home – The Cancer Genome Atlas – Cancer Genome – TCGA (n.d.). Available: http://cancergenome.nih.gov/. Accessed 26 Mar 2013
14. Imataka G, Arisaka O (2012) Chromosome analysis using Spectral Karyotyping (SKY). Cell Biochem Biophys 62:13–17. doi:10.1007/s12013-011-9285-2
15. Onkes W, Fredrik R, Micci F, Schönbeck BJ, Martin-Subero JI et al (2013) Breakpoint characterization of the der(19)t(11;19)(q13;p13) in the ovarian cancer cell line SKOV-3. Gene Chromosomes Cancer 52:512–522. doi:10.1002/gcc.22048
16. Wang L, Wenners A, Hilpert F, Fredrik R, Micci F et al (2014) Frequent translocations of 11q13.2 and 19p13.2 in ovarian cancer. Gene Chromosomes Cancer 53:447–453. doi:10.1002/gcc.22152
17. Kannan K, Coarfa C, Rajapakshe K, Hawkins SM, Matzuk MM et al (2014) CDKN2D-WDFY2 is a cancer-specific fusion gene recurrent in high-grade serous ovarian carcinoma. PLoS Genet 10:e1004216. doi:10.1371/journal.pgen.1004216
18. Cancer Genome Atlas Network (2012) Comprehensive molecular characterization of human colon and rectal cancer. Nature 487:330–337. doi:10.1038/nature11252
19. Seshagiri S, Stawiski EW, Durinck S, Modrusan Z, Storm EE et al (2012) Recurrent R-spondin fusions in colon cancer. Nature 488:660–664. doi:10.1038/nature11282
20. Nome T, Hoff AM, Bakken AC, Rognum TO, Nesbakken A et al (2014) High frequency of fusion transcripts involving TCF7L2 in colorectal cancer: novel fusion partner and splice variants. PLoS One 9:e91264. doi:10.1371/journal.pone.0091264
21. Salzman J, Gawad C, Wang PL, Lacayo N, Brown PO (2012) Circular RNAs are the predominant transcript isoform from hundreds of human genes in diverse cell types. PLoS One 7:e30733. doi:10.1371/journal.pone.0030733
22. Bienz M, Clevers H (2000) Linking colorectal cancer to Wnt signaling. Cell 103:311–320
23. Shinmura K, Kahyo T, Kato H, Igarashi H, Matsuura S et al (2014) RSPO fusion transcripts in colorectal cancer in Japanese population. Mol Biol Rep 41:5375–5384. doi:10.1007/s11033-014-3409-x
24. Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R et al (2005) Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science 310:644–648. doi:10.1126/science.1117679

Part VI
Other Aspects

Chapter 20
Pre-clinical Modelling of Chromosomal Translocations and Inversions

Katia Ruggero and Terence H. Rabbitts

Contents

20.1 Why It Is Important to Recapitulate Human Chromosomal Translocations in Preclinical Models 430
20.2 Strategies to Model Chromosomal Translocations and Translocation Genes by Transgenesis 430
20.2.1 Generation of Transgenic Mice by DNA Microinjection into Mouse Zygote Embryos 430
20.2.1.1 Transgenic Mouse Models of Leukaemia/Lymphoma 432
20.2.2 Haematopoietic Stem Cell Transplantation Models 434
20.2.2.1 Bone Marrow Transplantation Methodology 434
20.2.2.2 Bone Marrow Transplantation Models 434
20.3 Chromosomal Translocation Mimics Created by Gene Targeting 435
20.3.1 Gene Targeting of Mouse Embryonic Stem Cells 435
20.3.2 The Knock-in Mouse Chromosomal Translocation Mimics 435
20.3.3 The Translocator Mouse Model Recapitulates Human Chromosomal Translocations 437
20.3.4 The Invertor Mouse Model 438
20.4 Zebrafish as a Model System for Creating Chromosomal Translocations 439
20.5 New Methods for Possible Application to Chromosomal Translocation Modelling 440
References 441

Abstract Animal models of tumour formation induced by chromosomal translocations are unique tools to study the consequence and tumourigenic potential of these chromosomal abnormalities in a living animal. There are several approaches to generate mouse translocation models including microinjection into one-cell embryos of the translocation or fusion transgene, bone marrow transplantation models for leukaemia/lymphoma, and gene targeting by homologous recombination for specific expression in chimaeric mice using embryonic stem cells. Other model organisms

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such as zebrafish are also available as alternatives to mice. In addition, new gene editing technologies promise to facilitate rapid generation of parallel models of human chromosomal abnormalities.

Keywords Chromosomal translocations • Fusion genes • Leukaemia • Sarcoma • Preclinical models

20.1 Why It Is Important to Recapitulate Human Chromosomal Translocations in Preclinical Models

Animal models of tumour formation induced by chromosomal translocations or inversions are unique tools for obtaining information about the consequence and tumourigenic potential of these chromosomal abnormalities occurring in a living animal. In the context of this chapter, we will include all oncogene activations and gene fusions, including those that arise by inversions (for example in prostate cancer, see Chap. 17), under a generic classification of translocations for simplicity. Animal tumour models allow the dissection of the molecular mechanisms of growth control, cell death and differentiation driven by the translocation event, the study of the disease development from the earlier stages to the overt neoplasia, and the development of new treatment strategies. In particular the latter objective is critically important as a preclinical setting to test new drugs and new therapeutic approaches such as nanoparticle delivery [1]. The de novo models are superior to the much maligned xenograft approach used in drug development since transgenesis (all forms) start the tumour process from a single genetic change (i.e. the transgene) and may require additional mutations to fully manifest overt cancer whereas xenografts employ tumour cell lines or biopsies of fully fledged tumours.

There are several approaches to generate translocation gene models ranging from random chromosomal insertion of transgenes constructed to express activated genes and fusions or gene targeting for specific expression (these general options are summarized in Fig. 20.1). At the end of the chapter, we briefly review new gene editing technologies that promise to facilitate more rapid generation of parallel models of human chromosomal abnormalities.

20.2 Strategies to Model Chromosomal Translocations and Translocation Genes by Transgenesis

20.2.1 Generation of Transgenic Mice by DNA Microinjection into Mouse Zygote Embryos

Mice are the most widely used animal models for transgenic studies since they have several technical advantages. The mouse genome shows a high degree of conservation with the human genome: of approximately 22,000 human protein coding genes,

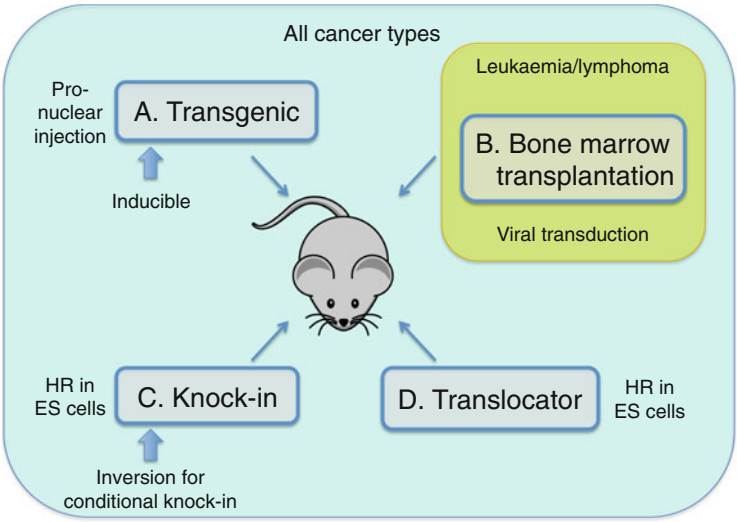

Fig. 20.1 General approaches to model chromosomal translocation and gene fusions. (**a**) Transgenic mouse models can be generated by microinjection of an expression cassette coding for the translocation or fusion gene. Variants of this system are inducible forms of transgenes. (**b**) Bone marrow transplantation: isolated bone marrow cells can be transduced with retroviral or lentiviral vectors harbouring the transgene and transplanted into a recipient mice to create a mouse model of leukaemia/lymphoma disease. (**c**, **d**) Chromosomal translocation mimics can be created by gene targeting in embryonic stem cells (*ES* cells) transfected with a vector designed to recombine via homologous recombination (*HR*) with the ES genome. Engineered ES cells can be injected into blastocysts to create chimaeric mice that in turn can be bred to give germline transmission of the targeted loci. In this strategy, knock-in (**c**) mice express the translocation protein as an endogenous gene. Conditional forms of knock-in can be made and the invertor model is fully conditional dependent on the transcriptional orientation of the knock-in gene. Complete recapitulation of the human chromosomal translocation event can be achieved by the translocator model (**d**) in which a de novo chromosomal translocation occurs via cre-*loxP* inter-chromosomal translocation. Inversions and deletions can also be obtained by application of cre-*loxP* recombination

about 18,000 have an identifiable mouse orthologue, with median 85 % sequence identity (Source: ensembl version 81, July 2015). Mice are readily available and have high reproductive rate. Moreover breeding strategies can combine different strains harbouring genetic alterations that create a versatile approach for the re-creation and study of human diseases.

A well-characterized approach to generate transgenic animals is the microinjection into pro-nuclei of one-cell embryos, first developed in the early 1980s where fertilized oocytes are surgically collected from a female donor mouse and exogenous DNA is micro-injected [2–5]. After exogenous DNA enters the nucleus, a random double strand breakage (DSB) occurs and chromosomal DNA and exogenous DNA are ligated by the non-homologous end joining (NHEJ). The DNA usually integrates at one genomic site with a copy number that can vary from one to several hundreds. With this technique, all the cells of the adult mouse will carry the transgene and it will be expressed in all cells in which the specific promoter is active. In

the following sections, we have discussed some examples, but not fully comprehensive, of the application of this technology to leukaemia/lymphoma development.

20.2.1.1 Transgenic Mouse Models of Leukaemia/Lymphoma

The first transgenic translocation gene mouse model was the *Eu-myc* mouse modelling the effect of *MYC* gene activation following chromosomal translocation in Burkitt B-cell lymphoma [6]. *MYC* translocation juxtaposes the *MYC* gene to one of the three immunoglobulin genes, leading to ectopic *MYC* expression [7]. The *Eμ-Myc* transgenic mice develop an aggressive pre-B or B cell leukaemia within 1 year of age. This model formally showed ectopic MYC is oncogenic but did not fully recapitulate Burkitt lymphoma phenotype, possibly due to heavy expression loads caused by the immunoglobulin enhancer.

The first chromosomal translocation discovered by cytogenetic analysis (the Philadelphia chromosome, see Chap. 7) was shown to create the fusion protein BCR-ABL1 [8], a constitutively active tyrosine kinase identified in chronic myelogenous leukaemia (CML). Several strategies have been employed to develop mouse models of the *BCR-ABL1* fusion gene using transgenic technology. An early model overexpressing the *BCR-ABL1* transgene under the control of the immunoglobulin heavy-chain enhancer or retroviral long terminal repeat developed T-cell lymphomas and neuro-ectodermal tumours [9]. Inducible transgenic models of *BCR-ABL1* expression were created using the tetracycline regulation system [10] where the transgene was controlled by an inducible tetracycline operator/repressor system. In these models, when expression of the *BCR-ABL1* transgene was controlled by induction through a tetracycline activator controlled from a MMTV-LTR (mammary tumour virus long terminal repeat) promoter [11], or from the CD34 promoter [12], pre B-cell acute lymphoblastic leukaemia or a myeloproliferative syndrome resembling thrombocythemia, respectively, occurred. In a similar inducible *BCR-ABL1* mouse model, the tetracycline activator was expressed under the control of the *TAL1/SCL* (stem cell leukaemia) gene 3′ enhancer and the transgenic mice developed full blown CML-like leukaemia, with a survival period of 4–10 weeks, characterized by neutrophilia, leukocytosis and splenomegaly. A critical finding emerging from *BCR-ABL1* inducible transgenic models was that the effect was reversible (by tetracycline) showing that the tumours were responsive only to BCR-ABL1 [11, 13].

T-cell acute leukaemia is characterized by many different recurrent chromosomal translocations (see Chap. 10) and amongst these are LMO2 translocations t(11;14)(p13;q11.2) and t(7;11)(q34;p13) [14]. Furthermore, LMO2 has been activated in four patients subjected to gene therapy for X-SCID correction, using an IL2Rγc-expressing retrovirus, resulting in T cell leukaemia [15, 16]. T-cell acute leukaemia has been modelled in transgenic mice using *LMO2* transgenes where *Lmo2* is expressed from *CD2*, *Thy1* or *metallothionein* promoters resulting in clonal T cell neoplasia [17–21], including mice in which the transgene was

controlled by the general transcription *metallothionein* promoter [21]. This strongly suggests that the oncogenic role of LMO2 is restricted to the T cell lineage. This contention is supported by observations about the role of LMO2 in a pre-leukaemias (pre-symptomatic) phase as abnormal expression of LMO2 has a profound effect on T cell differentiation within the thymus. It was discovered that a role for LMO2 in the asymptomatic phase was to cause a partial differentiation block at the stage of immature thymocytes (CD4; CD8 double negative (DN) cells) [19, 20]. In particular, the DN3 thymocyte subset (CD44-; CD25+) was relatively increased in *CD2-Lmo2* transgenic mice prior to their showing any leukaemia phenotype [22]. This thymocyte subset has an increased self-renewal capacity in a syngeneic transplantation assay [23]. These observations highlight an important element of transgenic models of human disease where it is possible to find and study asymptomatic, early stages of cancer development that is not possible for human LMO2-associated T-ALL since patients present with overt disease and the pre-leukaemic phase is not seen.

The reciprocal translocations involved *RUNX1* or *KMT2A*/*MLL* genes commonly occur in leukaemia (see Chaps. 8 and 11). The translocation t(8;21)(q22;q22) leads to the expression of the fusion protein RUNX1-RUNX1T1 (previously AML1-RUNX1T1). A knock-in mouse model for RUNX1-RUNX1T1 was first developed (for a discussion of knock-in technology, see Sect. 3.2), but expression of the fusion protein resulted in embryonic lethality. To bypass this, several strains of transgenic mice expressing RUNX1-RUNX1T1 have been generated, in particular an inducible transgene under the control of a tetracycline responsive element. However, leukaemias did not develop in these mice [24]. A conditional mouse model of AML with expression of *RUNX1-RUNX1T1* restricted to the haematopoietic compartment was produced using BM cells derived from a transgenic mouse expressing *RUNX1-RUNX1T1* under the control of a TET-ON system to reconstitute lethally irradiated recipient animals. Reconstituted mice developed long latency indolent myeloproliferative disease, and RUNX1-RUNX1T1 ablation leads to recovery from the phenotype [25]. Interestingly, the expression of the *ETV6-RUNX1* fusion gene (previously *TEL-AML1*; generated by the t(12;21)(p13;q22) chromosomal translocation) is not sufficient to induce leukaemogenesis in transgenic mouse models [26–28] even though the ETV6-RUNX1 fusion is the most commonly found in childhood acute lymphoblastic leukaemias (ALLs) (see Chap. 10).

The mixed lineage leukaemia (*KMT2A*) gene, located on chromosome 11, band q23, is a gene frequently involved in chromosomal translocations and is involved in the development of the whole range of haematological malignancies (see Chap. 11). Modelling these translocations has mainly been through the use of ES cell gene targeting methods (see below Sect. 3) but a transgenic mouse line overexpressing an *KMT2A-AFF1* fusion gene has been studied and the transgenic mice developed B-cell lymphoma in the spleen, liver and lungs with long latency, while concomitant mutant *KRAS* accelerated the leukaemia onset [29]. However, this model does not provide a recapitulation of the early onset and phenotype of childhood disease associated with a *KMT2A* rearrangement.

20.2.2 Haematopoietic Stem Cell Transplantation Models

20.2.2.1 Bone Marrow Transplantation Methodology

A method that can be applied to murine haematopoietic stem cells (HSCs) is their transduction ex vivo using lentiviral or retroviral vectors harbouring a transgene of interest (e.g. a fusion gene) [30, 31]. After transplantation of these infected donor cells into lethally irradiated recipient mice, the transduced HSCs repopulate the recipient bone marrow (BM) and stably reconstitute their entire haematopoietic system, detectable in the recipient mouse after about 1 month post-transplantation [32, 33]. This technique provides a useful tool to dissect the influence of a transgene expression in the haematopoietic and tumour development. Compared to the generation of a transgenic mouse model, the bone marrow reconstitution approach has the advantage of being rapid and efficient, the exogenous gene expression is confined to the haematopoietic lineage and donor cells can be transplanted to several recipient mice, with the disadvantage of the non-inheritance of the transgene. Further the co-expression of the transgene with a tracking gene, such as that encoding green fluorescent protein, allows the donor cell engraftment efficiency to be monitored.

20.2.2.2 Bone Marrow Transplantation Models

Mouse bone marrow transplantation assays were first employed to create mouse models of the *BCR-ABL1* fusion found in Philadelphia chromosome-positive CML. The retroviral transduction of a *BCR-ABL1* transgene into BM cells, followed by transplantation into irradiated recipient mice, proved a faithful model of human CML [34–36]. However, transduction of other gene fusions have not been such a good reproduction of human leukaemia. *RUNX1-RUNX1T1* expressing lentiviruses transduced into murine HSCs or total BM followed by BM transplantation to recipient mice resulted in haematological abnormalities but not to frank leukaemia [37–39]. Conversely, transduction of a truncated isoform of *RUNX1-RUNX1T1* (*RUNX1-ETO9a*) into mouse foetal liver, followed by transplantation, caused spontaneous full-blown leukaemia without the need of any other secondary mutations [40]. Similarly, models of *ETV6-RUNX1* using mouse BM transplantation showed impairment of normal haematopoietic differentiation and B cell differentiation block, but no leukaemic development [27, 28]. In another study, reconstituted mice develop leukaemia with low incidence, increased by concomitant *Cdkn2a* ($p16^{INK4a}p19^{ARF)}$ loss, but the phenotype did not represent the B-cell precursor acute lymphoblastic leukaemia (BCP-ALL) associated with *ETV6-RUNX1* translocation [26]. The mixed success of BM transplantation studies to recapitulate the human cancers may reflect many different short-comings of the technique but the findings suggest hidden levels of complexity in the aetiology of these human leukaemias.

20.3 Chromosomal Translocation Mimics Created by Gene Targeting

20.3.1 Gene Targeting of Mouse Embryonic Stem Cells

The transgenic methods (DNA microinjection or viral transduction) have the disadvantage of non-specific insertion sites in the genome of the introduced DNA. New gene targeting methods have been developed to allow the targeting to specific chromosomal locations [41–43] and have been of particular utility to recreate de novo chromosomal translocations involving two different chromosomal loci in the genome and conditional translocation mimics [2, 44–47]. The strategies that can be used to implement gene targeting to create mouse models of chromosomal translocations are summarized in Fig. 20.2c, d.

The general method of gene targeting involves using mouse pluripotent embryonic stem (ES) cells transfected with a vector designed to recombine via homologous recombination (HR) at specific sites with the ES genome. When a targeted ES clone is identified, it can be injected into blastocysts for the creation of chimaeric mice carrying cells of donor ES origin and recipient blastocyst origin and these implanted into the uterus of a pseudo-pregnant foster mother to allow the embryos to come to term. Temporal gene expression can be achieved by direct homologous recombination into a tissue or cell-specific gene (knock-in discussed below).

The outcome of the HR events can be amended by utilizing site-specific recombinases (commonly Cre/*loxP* and the Flp/*frt*) to create conditional forms of genetic modification (i.e. where the effect of the gene alteration is restricted spatially and temporally) lending a further degree of sophistication to the mouse pre-clinical cancer models [46, 48]. The Cre or Flp recombinases recognize short nucleotide stretches located in genomes and can delete, invert or translocate between chromosomes. Thus a spatial, conditional genetic modification is one where a transcriptional stop signal, flanked by recombinase recognition sequences, is inserted near a gene and can be removed by expressing recombinase [49]. Alternatively, genes can be flanked by recombinase recognition sequences and activated by recombinase (e.g. the invertor model discussed below) or inter-chromosomal translocation can occur between recombinase recognition sequences located on non-homologous chromosomes (the translocator model discussed below).

20.3.2 The Knock-in Mouse Chromosomal Translocation Mimics

The homologous recombination knock-in concept (Fig. 20.2a) was specifically developed for gene fusion in 1996 by the fusion of the human *AF9* cDNA into the mouse *Kmt2a* gene [50] to recapitulate the effect of the t(9;11)(p21.3;q23.3) human translocation that forms the KMT2A-MLLT3 fusion protein. Chimaeric and first

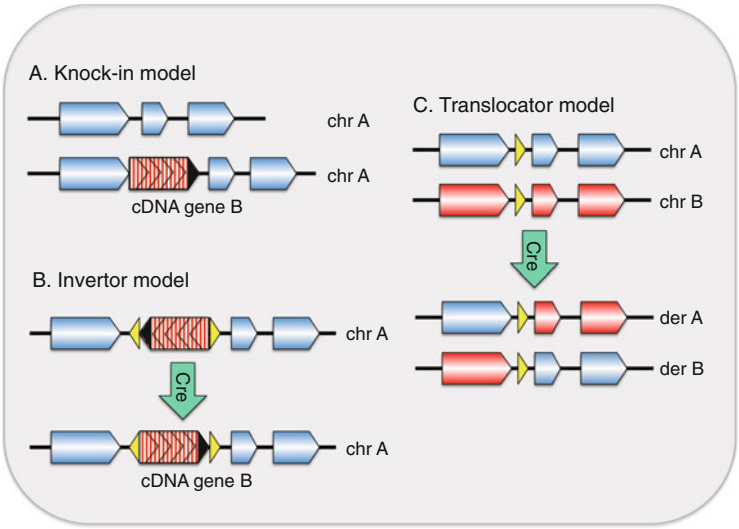

Fig. 20.2 Generation of chromosomal translocation mimics via homologous recombination in ES cells. (**a**) In the knock-in mouse model, the cDNA sequence of a gene (depicted as gene B) is knocked-in at the appropriate exon of the target gene (depicted in *blue*) in order to create a fusion gene. The inserted cDNA is fused in-frame with the coding sequences of the host gene, thereby making a fusion product with that gene and the cDNA sequence brings the new translation termination codon. (**b**) The invertor mouse model is based on the knock-in concept but is a fully conditional knock-in approach. A cDNA flanked by inverted *loxP* sites is knocked-in with inverted transcriptional orientation to the intron of the target gene and thus is initially silent. Cre-mediated recombination inverts the cDNA cassettes placing the sequence in the correct transcriptional orientation to cause transcription of the fusion gene. (**c**) The methods in *panels a* and *b* do not fully reproduce the effects of inter-chromosomal translocation as they mimic only one of the derivative chromosomes (these methods are applicable to intra-chromosomal inversion or deletion, although in the former two potential products could be important). The translocator mouse model fully recapitulates inter-chromosomal translocations as the method creates a de novo reciprocal chromosomal translocation using the recombinase-mediated recombination. *LoxP* sites are inserted in the introns of the genes involved in the chromosomal translocation, corresponding to the intronic site where a human chromosomal translocation breakpoint occurs. After activation of Cre expression, inter-chromosomal translocation can occur to produce two derivative (der) translocation chromosomes. Key: LoxP site: *yellow triangle*. chr: chromosome. exons: *blue* and *red boxes*. cDNA: *striped boxes* and the direction of transcription is indicated by the *black chevrons*. Stop codon: *black triangle*

generation heterozygous mice for the knock-in fusion developed AML from the sixth month of age, recapitulating the human disease. The targeted mice showed increased myeloid cell proliferation in the bone marrow from birth, where presumably secondary mutations occurred to contribute to the overt disease.

The knock-in approach lacks cell type and temporal control, since the knock-in gene (e.g. KMT2A-MLLT3) is expressed under the control of the targeted gene promoter and therefore the fusion protein will be expressed in every cell in which the endogenous promoter is active. If the fusion protein is expressed in the prenatal

stage and interferes with normal mouse development, the knock-in approach can result in embryonic lethality, such as in the case of *KMT2A-AFF1* fusion protein [51] or *RUNX1-RUNX1T1* [52]. In another study, a viable knock-in model of the *KMT2A-AFF1* fusion [53] yielded mice that developed long latency B cell lymphomas where clonal tumours appeared suggesting that secondary mutations are required for *KMT2A-AFF1* tumourigenesis. An amendment to the knock-in approach was developed for a conditional *RUNX1-RUNX1T1* model using a LoxP-STOP insertion [54] so that the knock-in strain could be bred with a Cre-expressing strain under the control of an inducible promoter. While this strategy obviated the embryonic lethality of the RUNX1-RUNX1T1 protein, RUNX1-RUNX1T1 expression was not able to induce leukaemia alone, but only in co-operation with other mutations induced by ENU treatment.

The first knock-in mouse model of *ETV6-RUNX1* was designed to induce the expression of the fusion protein under the control of the endogenous *ETV6* promoter. A truncated human *RUNX1* cDNA was targeted into exon 6 of the *ETV6* gene. A *loxP* flanked transcriptional stop site was present upstream of the *RUNX1* cDNA and the excision of the stop site was achieved during early embryogenesis by breeding *RUNX1* knock-in mice with a *Gata1-Cre* mouse strain. *ETV6-RUNX1* knock-in mice, however, failed to develop leukaemia even though normal B cell development was impaired [55]. Coupling a *ETV6-RUNX1* knock-in with the induction of secondary mutations produced by the Sleeping Beauty transposase derived mice that developed leukaemia with a BCP-ALL phenotype in 20 % of cases [56].

The development of models of sarcoma translocations have been impaired by a number of technical issues, including embryonic lethality of mice expressing the fusion proteins produced in these human cancers (see the Chaps. 14 and 15 for a full description of sarcoma translocations). One successful model of synovial sarcoma has been developed [57] where the *SS18-SSX2* fusion was gene targeted into the *ROSA26* locus (ubiquitously expressed) with a loxP-STOP preventing *SYT-SSX* expression. The activation of the *SYT-SSX* gene was achieved using Myf5-Cre and these mice developed a synovial-type sarcoma. This strategy is potentially applicable to other forms of sarcoma where the intriguing variance of translocation fusions occurs.

20.3.3 The Translocator Mouse Model Recapitulates Human Chromosomal Translocations

Mouse models with conditional gene expression avoid limitations due to embryonic lethality by permitting expression of the fusion protein in specific cell types and at specific stages of cellular differentiation. The translocator mouse model (Fig. 20.2b) exploits the capability of Cre-mediated recombination to mediate chromosomal translocations in vivo as shown in ES cells [58, 59]. This approach represents an ideal model to recapitulate a human chromosomal translocation since it permits

creation of de novo translocations in vivo without the need of selection. Further, the translocations naturally create both reciprocal translocation alleles and thus has important consequences for those rearrangements where there are issues about the involvement of both derivative translocation chromosomes in the development and/or maintenance of the cancer.

Translocators were first made by *loxP* site insertion into *Kmt2a* and *Mllt3* [60] in order to mimic the human translocation t(9;11)(p21.3;q23.3) and *Aml1* (*Runx1*) and *RUNX1T1* to mimic the translocation t(8;21)(q22;q22.12) [61]. Tumours arose in translocator mice that were mimics of the human t(11;19)(q23;p13.3) fusing *Kmt3a* with *Mllt1* by Cre expressed from the *Lmo2* gene [62]. This revealed an interesting apparent feature of the KMT2A-MLLT1 fusion, namely that a leukocytosis occurred that seemed to be dependent only on KMT2A-MLLT1 since the ensuing primary neoplasias were lethal to the mice, but could not be transplanted [62].

The translocator model can mimic cell-specific chromosomal translocations by using Cre recombinase expressed from cell-specific genes. In this way, the *Kmt2a-MllT1* and *Kmt2a-Mllt3* fusions were compared using *Cre* expressed from pluripotent progenitors (*Lmo2-Cre*) or committed T cells (*Lck-Cre*) [63]. While *Kmt2a-Mllt3* translocations only gave myeloid tumours and did not seem penetrant in T cells, *Kmt2a-MllT1* translocations occurring in the T cell lineage led to the development of either myeloid and T-cell tumours. This showed that KMT2A-MLLT1 can dictate lineage reassignment from the T-cell to the myeloid compartment demonstrating the role of KMT2A-fusion proteins and also the lineage plasticity of T cells, even after RAG-mediated T cell receptor rearrangement [63].

A drawback of the translocator model is cost and time. The method requires three targeted alleles (the *Cre* knock-in and the two *loxP* donor chromosomes) and cross breeding of mice to generate carriers of the three genes. A recently developed adaptation of the translocator method, the fast throughput chimaeric system, generates multiple, sequentially targeted ES cells used to develop cohorts of translocator chimaeras [64]. This method reduces the time for generation of translocator mice and the number of mice needed. For instance, the *Kmt2a-Mllt1*chimaera translocator system produced neoplasias of the same phenotype and rate of appearance in the chimaeric mice as in the germline translocators.

20.3.4 The Invertor Mouse Model

A requirement of the translocator model is that the mouse genes to be translocated must be in the same transcriptional orientation with respect to the chromosome centromere, to avoid the formation of dicentric and acentric derivatives chromosomes after the translocation event. Some cases (e.g. *Kmt2a* and *Aff1*) are therefore unsuitable for the translocator approach. The invertor method is a fully conditional knock-in approach devised to apply to situations where the translocator method could not be applied [65] but only produces one of the translocation chromosome mimics since it relies on an amended knock-in strategy. For the invertor method, a

cDNA, flanked by *loxP* sites, is knocked-in by homologous recombination in ES cells, into the intron of the target gene but with an inverted transcriptional orientation and therefore it is transcriptionally silent (Fig. 20.2c). After Cre expression, the knocked-in gene is inverted and the fusion gene created to produce the fusion protein in a cell-specific way, depending on the expression pattern of Cre. The first invertor mouse expressed the *EWSR1-ERG* fusion gene controlled by *Rag1-Cre* [65, 66] resulting in T-cell leukaemia [66]. A similar application was used for modelling the *Kmt2a-AFF1* fusion [67]. The mice carrying the *Kmt2a-AFF1* invertor cassette were crossed with a Cre expressing strain under the control of B cell or T cell promoters, the majority of which progressed to long latency, mature diffuse large B-cell lymphomas, providing further evidence of the instructive model of KMT2A-fusion associated leukaemias [67]. However, it is noted once again that attempts to model human B cell tumours in mice induced a different phenotype to that observed in human tumours.

20.4 Zebrafish as a Model System for Creating Chromosomal Translocations

As an alternative to mouse modelling, Zebrafish provides several unique features, such as the possibility of large-scale genetic and chemical screens with relatively low cost and relatively minimal space requirements coupled to fast embryonic development. Moreover, transparent embryos generated by the external fertilization permit in vivo imaging of fluorescent tagged cells. Zebrafish are particularly suitable for the generation of haematological malignancy models since developmental stages and genetic patterns are highly conserved with mammals [68]. However, thus far, relatively little success has been achieved.

Several Zebrafish transgenic or knock-in models have been generated as human chromosomal translocation mimics for fusion proteins, such as *ETV6-JAK2a* [69], *EWSR1-FLI1* [70], *NUP98-HOXA9* [71], *RUNX1-RUNX1T1* [72, 73] and *ETV6-RUNX1* [74]. Transgenic Zebrafish expressing *MYC* under the control of the Rag2 promoter represent a model of aggressive T-ALL able to recapitulate some aspects of the human disease [75]. In a conditional version of the model, *MYC* expression was induced after the animals reached sexual maturity, leading to I expression and development of T-ALL with a delayed onset [76].

Transgenic expression of the *ETV6-RUNX1* fusion protein was induced ubiquitously under the control of *Xenopus* elongation factor 1 or the Zebrafish β-actin promoters, and in B and T cell lymphoid progenitors using the Zebrafish *Rag2* promoter. Fish with ubiquitous *ETV6-RUNX1* expression showed progenitor cell expansion and a B-cell differentiation arrest that evolved into B-cell leukaemia but only in a low proportion of the fish [74], whereas ETV6-*RUNX1-Rag2* fish did not develop leukaemia or progenitor cell expansion. The ubiquitous overexpression of the *RUNX1-RUNX1T1* fusion protein leads to early embryonic lethality in fish, as it

does in mice, while conditional activation of *RUNX1-RUNX1T1* in Zebrafish embryos caused reprogramming of haematopoietic progenitor cells into granulocytic cells [72] but leukaemias again failed to manifest.

20.5 New Methods for Possible Application to Chromosomal Translocation Modelling

The vast majority of transgenic mice produced so far relied on the use of microinjection of expression constructs into fertilized oocytes or on homologous recombination in mouse ES cells. There are advantages and limitations to these methods as outlined above. Among limitations of the transgenic methods is the random insertion of the DNA constructs that can lead to anomalies or the size of the expression construct designed to emulate the expression. Bacterial artificial chromosomes (BACs) are a category of vectors that can harbour large fragments of genomic DNA (up to 300 kb), that can also be easily modified and sub-cloned using homologous recombination in *E. coli* recombineering [77–79] BACs are vehicles that can allow the insertion of complete genes sequences together with natural regulatory elements, for example a translocation fusion gene. A limitation is again that, simplistically, only one translocation allele can be studied per BAC clone.

An additional set of tools that are potentially useful for chromosomal translocation mimics are the gene editing methods. Initially, the use of engineered sequence-specific endonucleases has emerged as a valuable alternative for the generation of targeted mutations. Two classes of engineered endonucleases can be used to manipulate the animal genome: zinc-finger nucleases (ZFNs) [80] and transcription activator-like effector nucleases (TALENs) [81, 82]. After binding of a pair of endonucleases to the target sites in the genome, the cleavage domains heterodimerize to introduce a double-strand break followed by non-homologous end-joining [83–85]. Gene insertions have been achieved using TALENS [86] and this could be applied to knocking-in translocation genes.

An analogous technology that holds great promise for mimicking the consequences of chromosomal translocations is the RNA-guided nuclease CRISPR/CAS9 system. This is based on the activity of clustered regularly interspaced short palindromic repeat (CRISPR) genes, derived from the endogenous adaptive immune system of bacteria and archea [87–89]. CRISPR associated (Cas) proteins are endonucleases that form an RNA/protein complex together with a guide RNA (gRNA) to direct the CRISPR complex to the corresponding nucleic acid with which it shares a short stretch of homology, and mediates its degradation by the Cas protein. It is also possible to efficiently perform CRISPR/Cas-directed targeting directly into the one-cell embryo, by co-injecting a Cas9 expressing mRNA and synthetic gRNAs. Jaenisch and collaborators targeted with high efficiency mouse ES cells by using several gRNAs simultaneously and generated mice carrying disruptions in multiple genes [90]. The CRISPR/Cas system has also be applied to knock-in studies, for

instance a study used the CRISPR/Cas9 mediated homology independent DSBs repair to introduce donor DNA of up to 5.7 kb into the Zebrafish genome [91]. Further, recent work inducing simultaneous breaks on non-homologous and homologous chromosomes using zinc finger nucleases, TALENS or CRISPR/cas9 has shown that inter-chromosomal translocations and intra-chromosomal inversions can occur [92–95]. While these events are not conditional, they demonstrate that sequence specified double strand breaks can result in exchanges, and suggest that these systems could be adapted to generate tissue-specific chromosomal translocations.

Taken together, adaptations of the gene editing technologies could produce rapid and efficient chromosomal translocation mimics that will more faithfully recapitulate the corresponding human cancer mutations and, with the use of chimaeric mice [50], also fulfil an aim of the 3Rs ethical framework for reduction in animal numbers in these crucial pre-clinical cancer models.

References

1. Davis ME, Chen ZG, Shin DM (2008) Nanoparticle therapeutics: an emerging treatment modality for cancer. Nat Rev Drug Discov 7(9):771–782
2. Belizário JE, Akamini P, Wolf P, Strauss B, Xavier-Neto J (2012) New routes for transgenesis of the mouse. J Appl Genet 53(3):295–315, Review
3. Hofker MH, Breuer M (1998) Generation of transgenic mice. Methods Mol Biol 110:63–78, Review
4. Sigmund CD (1993) Major approaches for generating and analyzing transgenic mice. Hypertension 22(4):599–607
5. Krimpenfort P, Berns A (1987) Gene transfer into mammalian embryos. Hum Reprod 2(4):333–339, Review
6. Adams JM, Harris AW, Pinkert CA, Corcoran LM, Alexander WS, Cory S, Palmiter RD, Brinster RL (1985) The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. Nature 318(6046):533–538
7. Rabbitts TH (1994) Chromosomal translocations in human cancer. Nature 372:143–149
8. Hermans A, Heisterkamp N, von Linden M, van Baal S, Meijer D et al (1987) Unique fusion of bcr and c-abl genes in Philadelphia chromosome positive acute lymphoblastic leukemia. Cell 51:33–40
9. Adams JM, Harris AW, Strasser A, Ogilvy S, Cory S (1999) Transgenic models of lymphoid neoplasia and development of a pan-hematopoietic vector. Oncogene 18(38):5268–5277, Review
10. Stieger K, Belbellaa B, Le Guiner C, Moullier P, Rolling F (2009) In vivo gene regulation using tetracycline-regulatable systems. Adv Drug Deliv Rev 61(7–8):527–541. doi:10.1016/j.addr.2008.12.016, Epub 2009 Apr 23. Review
11. Huettner CS, Zhang P, Van Etten RA, Tenen DG (2000) Reversibility of acute B-cell leukaemia induced by BCR-ABL1. Nat Genet 24:57–60
12. Huettner CS, Koschmieder S, Iwasaki H et al (2003) Inducible expression of BCR/ABL using human CD34 regulatory elements results in a megakaryocytic myeloproliferative syndrome. Blood 102:3363–3370
13. Koschmieder S, Gottgens B, Zhang P, Iwasaki-Arai J, Akashi K et al (2005) Inducible chronic phase of myeloid leukemia with expansion of hematopoietic stem cells in a transgenic model of BCR-ABL leukemogenesis. Blood 105(1):324–334

14. Boehm T, Foroni L, Kaneko Y, Perutz MF, Rabbitts TH (1991) The rhombotin family of cysteine-rich LIM-domain oncogenes: distinct members are involved in T-cell translocations to human chromosomes 11p15 and 11p13. Proc Natl Acad Sci U S A 88(10):4367–4371
15. Hacein-Bey-Abina S, Garrigue A, Wang GP, Soulier J, Lim A et al (2008) Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. J Clin Invest 118(9):3132–3142
16. Howe SJ, Mansour MR, Schwarzwaelder K, Bartholomae C, Hubank M et al (2008) Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. J Clin Invest 118(9):3143–3150
17. Fisch P, Boehm T, Lavenir I, Larson T, Arno J et al (1992) T-cell acute lymphoblastic lymphoma induced in transgenic mice by the RBTN1 and RBTN2 LIM-domain genes. Oncogene 7:2389–2397
18. Larson R, Fisch P, Larson TA, Lavenir I, Langford T et al (1994) T cell tumours with disparate phenotypes develop with long latency in mice transgenic for rbtn2. Oncogene 9:3675–3681
19. Larson RC, Osada H, Larson TA, Lavenir I, Rabbitts TH (1995) The oncogenic LIM protein Rbtn2 causes thymic developmental aberrations that precede malignancy in transgenic mice. Oncogene 11:853–862
20. Larson RC, Lavenir I, Larson TA, Baer R, Warren AJ et al (1996) Protein dimerisation between Lmo2 (Rbtn2) and Tal1 alters thymocyte development and potentiates T cell tumorigenesis in transgenic mice. EMBO J 15:1021–1027
21. Neale GA, Rehg JE, Goorha RM (1995) Ectopic expression of rhombotin-2 causes selective expansion of the thymus and T-cell tumours in transgenic mice. Blood 86:3060–3071
22. McCormack MP, Forster A, Drynan LF, Pannell R, Rabbitts TH (2003) The LMO2 T-cell oncogene is activated via chromosomal translocations or retroviral insertion during gene therapy but has no mandatory role in normal T-cell development. Mol Cell Biol 23(24):9003–9013
23. McCormack MP, Young LF, Vasudevan S, de Graaf CA, Codrington R et al (2010) The Lmo2 oncogene initiates leukemia in mice by inducing thymocyte self-renewal. Science 327(5967):879–883
24. Rhoades KL, Hetherington CJ, Harakawa N, Yergeau DA, Zhou L et al (2000) Analysis of the role of AML1-ETO in leukemogenesis, using an inducible transgenic mouse model. Blood 96(6):2108–2115
25. Cabezas-Wallscheid N, Eichwald V, de Graaf J, Löwer M, Lehr HA et al (2013) Instruction of haematopoietic lineage choices, evolution of transcriptional landscapes and cancer stem cell hierarchies derived from an AML1-ETO mouse model. EMBO Mol Med 5(12):1804–1820
26. Bernardin F, Yang Y, Cleaves R, Zahurak M, Cheng L et al (2002) TEL-AML1, expressed from t(12;21) in human acute lymphocytic leukemia, induces acute leukemia in mice. Cancer Res 62(14):3904–3908
27. Tsuzuki S, Seto M, Greaves M, Enver T (2004) Modeling first-hit functions of the t(12;21) TEL-AML1 translocation in mice. Proc Natl Acad Sci U S A 101(22):8443–8448
28. Fischer M, Schwieger M, Horn S, Niebuhr B, Ford A et al (2005) Defining the oncogenic function of the TEL/AML1 (ETV6/RUNX1) fusion protein in a mouse model. Oncogene 24(51):7579–7591
29. Tamai H, Miyake K, Takatori M, Miyake N, Yamaguchi H et al (2011) Activated K-Ras protein accelerates human MLL/AF4-induced leukemo-lymphomogenicity in a transgenic mouse model. Leukemia 25(5):888–891
30. Chang AH, Sadelain M (2007) The genetic engineering of hematopoietic stem cells: the rise of lentiviral vectors, the conundrum of the ltr, and the promise of lineage-restricted vectors. Mol Ther 15(3):445–456, Epub 2007 Jan 16. Review
31. Haviernik P, Zhang Y, Bunting KD (2008) Retroviral transduction of murine hematopoietic stem cells. Methods Mol Biol 430:229–241
32. Miller CL, Eaves CJ (1997) Expansion in vitro of adult murine hematopoietic stem cells with transplantable lympho-myeloid reconstituting ability. Proc Natl Acad Sci U S A 94:13648–13653

33. Morita Y, Ema H, Yamazaki S, Nakauchi H (2006) Non-side-population hematopoietic stem cells in mouse bone marrow. Blood 108:2850–2856
34. Van Etten RA (2001) Retroviral transduction models of Ph+ leukemia: advantages and limitations for modeling human hematological malignancies in mice. Blood Cells Mol Dis 27(1):201–205
35. Daley GQ, Van Etten RA, Baltimore D (1990) Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome. Science 247(4944):824–830
36. Kelliher MA, McLaughlin J, Witte ON, Rosenberg N (1990) Induction of a chronic myelogenous leukemia-like syndrome in mice with v-abl and BCR/ABL. Proc Natl Acad Sci U S A 87(17):6649–6653
37. de Guzman CG, Warren AJ, Zhang Z, Gartland L, Erickson P et al (2002) Hematopoietic stem cell expansion and distinct myeloid developmental abnormalities in a murine model of the AML1-ETO translocation. Mol Cell Biol 22(15):5506–5517
38. Schwieger M, Lohler J, Friel J, Scheller M, Horak I et al (2002) AML1-ETO inhibits maturation of multiple lymphohematopoietic lineages and induces myeloblast transformation in synergy with ICSBP deficiency. J Exp Med 196:1227–1240
39. Grisolano JL, O'Neal J, Cain J, Tomasson MH (2003) An activated receptor tyrosine kinase, TEL/PDGFbetaR, cooperates with AML1/ETO to induce acute myeloid leukemia in mice. Proc Natl Acad Sci U S A 100(16):9506–9511
40. Yan M, Kanbe E, Peterson LF, Boyapati A, Miao Y et al (2006) A previously unidentified alternatively spliced isoform of t(8;21) transcript promotes leukemogenesis. Nat Med 12(8):945–949
41. Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292(5819):154–156
42. Smithies O, Gregg RG, Boggs SS, Koralewski MA, Kucherlapati RS (1985) Insertion of DNA sequences into the human chromosomal beta-globin locus by homologous recombination. Nature 317:230–234
43. Thomas KR, Capecchi MR (1987) Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. Cell 51(3):503–512
44. Bouabe H, Okkenhaug K (2013) Gene targeting in mice: a review. Methods Mol Biol 1064:315–336. doi:10.1007/978-1-62703-601-6_23
45. Mortensen R (2006) Overview of gene targeting by homologous recombination. Curr Protoc Mol Biol Nov; Chapter 23:Unit 23.1. Review
46. Van der Weyden L, Adams DJ, Bradley A (2002) Tools for targeted manipulation of the mouse genome. Physiol Genomics 11:133–164
47. Heyer W-D, Ehmsen LT, Liu J (2010) Regulation of homologous recombination in eukaryotes. Ann Rev Genet 44:113–139
48. Misra RP, Duncan SA (2002) Gene targeting in the mouse: advances in introduction of transgenes into the genome by homologous recombination. Endocrine 19(3):229–238, Review
49. Sauer B (1998) Inducible gene targeting in mice using the Cre/lox system. Methods 14(4):381–392, Review
50. Corral J, Lavenir I, Impey H, Warren AJ, Forster A et al (1996) An Mll-AF9 fusion gene made by homologous recombination causes acute leukemia in chimeric mice: a method to create fusion oncogenes. Cell 85:853–861
51. Lobato MN, Metzler M, Drynan L, Forster A, Pannell R et al (2008) Modeling chromosomal translocations using conditional alleles to recapitulate initiating events in human leukemias. J Natl Cancer Inst Monogr 39:58–63
52. Yergeau DA, Hetherington CJ, Wang Q, Zhang P, Sharpe AH et al (1997) Embryonic lethality and impairment of haematopoiesis in mice heterozygous for an AML1-ETO fusion gene. Nat Genet 15:303–306
53. Chen W, Li Q, Hudson WA, Kumar A, Kirchhof N, Kersey JH (2006) A murine Mll-AF4 knock-in model results in lymphoid and myeloid deregulation and hematologic malignancy. Blood 108(2):669–677, Epub 2006 Mar 21

54. Higuchi M, O'Brien D, Kumaravelu P, Lenny N, Yeoh EJ et al (2002) Expression of a conditional AML1-ETO oncogene bypasses embryonic lethality and establishes a murine model of human t(8;21) acute myeloid leukemia. Cancer Cell 1(1):63–74
55. Schindler JW, Van Buren D, Foudi A, Krejci O, Qin J et al (2009) TELAML1 corrupts hematopoietic stem cells to persist in the bone marrow and initiate leukemia. Cell Stem Cell 5(1):43–53
56. Van der Weyden L, Giotopoulos G, Rust AG, Matheson LS, van Delft FW et al (2011) Modeling the evolution of ETV6-RUNX1-induced B-cell precursor acute lymphoblastic leukemia in mice. Blood 118(4):1041–1051
57. Haldar M, Hancock JD, Coffin CM, Lessnick SL, Capecchi MR (2007) A conditional mouse model of synovial sarcoma: insights into a myogenic origin. Cancer Cell 11(4):375–388
58. Van Deursen J, Fornerod M, Van Rees B, Grosveld G (1995) Cre-mediated site-specific translocation between nonhomologous mouse chromosomes. Proc Natl Acad Sci U S A 92(16):7376–7380
59. Smith AJ, De Sousa MA, Kwabi-Addo B, Heppell-Parton A, Impey H et al (1995) A site-directed chromosomal translocation induced in embryonic stem cells by Cre-loxP recombination. Nat Genet 9(4):376–385, Erratum in: Nat Genet 1996 Jan; 12(1):110
60. Collins EC, Pannell R, Simpson EM, Forster A, Rabbitts TH (2000) Inter-chromosomal recombination of Mll and Af9 genes mediated by cre-loxP in mouse development. EMBO Rep 1:127–132
61. Buchholz F, Refaeli Y, Trumpp A, Bishop JM (2000) Inducible chromosomal translocation of AML1 and ETO genes through Cre/loxP-mediated recombination in the mouse. EMBO Rep 1:133–139
62. Forster A, Pannell R, Drynan LF, McCormack M, Collins EC et al (2003) Engineering de novo reciprocal chromosomal translocations associated with Mll to replicate primary events of human cancer. Cancer Cell 3(5):449–458
63. Drynan LF, Pannell R, Forster A, Chan NM, Cano F et al (2005) Mll fusions generated by Cre- loxP -mediated de novo translocations can induce lineage reassignment in tumorigenesis. EMBO J 24(17):3136–3146
64. Chambers JS, Tanaka T, Brend T, Ali H, Geisler NJ et al (2014) Sequential gene targeting to make chimeric tumor models with de novo chromosomal abnormalities. Cancer Res 74(5):1588–1597
65. Forster A, Pannell R, Drynan LF, Codrington R, Daser A et al (2005) The invertor knock-in conditional chromosomal translocation mimic. Nat Methods 2(1):27–30
66. Codrington R, Pannell R, Forster A, Drynan LF, Daser A et al (2005) The Ews-ERG fusion protein can initiate neoplasia from lineage committed haematopoietic cells. Publ Libr Sci Biol 3(8):e242
67. Metzler M, Forster A, Pannell R, Arends MJ, Daser A et al (2006) A conditional model of MLL-AF4 B-cell tumourigenesis using invertor technology. Oncogene 25(3):3093–3103
68. Jing L, Zon LI (2011) Zebrafish as a model for normal and malignant hematopoiesis. Dis Model Mech 4(4):433–438. doi:10.1242/dmm.006791, Review
69. Onnebo SM, Condron MM, McPhee DO, Lieschke GJ, Ward AC (2005) Hematopoietic perturbation in zebrafish expressing a tel-jak2a fusion. Exp Hematol 33(2):182–188
70. Embree LJ, Azuma M, Hickstein DD (2009) Ewing sarcoma fusion protein EWSR1/FLI1 interacts with EWSR1 leading to mitotic defects in zebrafish embryos and human cell lines. Cancer Res 69(10):4363–4371
71. Forrester AM, Grabher C, McBride ER, Boyd ER, Vigerstad MH et al (2011) NUP98-HOXA9-transgenic zebrafish develop a myeloproliferative neoplasm and provide new insight into mechanisms of myeloid leukaemogenesis. Br J Haematol 155(2):167–181
72. Yeh JR, Munson KM, Chao YL, Peterson QP, Macrae CA et al (2008) AML1-ETO reprograms hematopoietic cell fate by downregulating scl expression. Development 135:401–410
73. Yeh JR, Munson KM, Elagib KE, Goldfarb AN, Sweetser DA et al (2009) Discovering chemical modifiers of oncogene-regulated hematopoietic differentiation. Nat Chem Biol 5(4):236–243. doi:10.1038/nchembio.147, Epub 2009 Jan 26

74. Sabaawy HE, Azuma M, Embree LJ, Tsai HJ, Starost MF et al (2006) TEL-AML1 transgenic zebrafish model of precursor B cell acute lymphoblastic leukemia. Proc Natl Acad Sci U S A 103(41):15166–15171
75. Langenau DM, Traver D, Ferrando AA, Kutok JL, Aster JC, Kanki JP, Lin S, Prochownik E, Trede NS, Zon LI et al (2003) Myc-induced T cell leukemia in transgenic zebrafish. Science 299:887–890
76. Feng H, Langenau DM, Madge JA, Quinkertz A, Gutierrez A, Neuberg DS, Kanki JP, Look AT (2007) Heat-shock induction of T-cell lymphoma/leukaemia in conditional Cre/lox-regulated transgenic zebrafish. Br J Haematol 138:169–175
77. Narayanan K, Chen Q (2011) Bacterial artificial chromosome mutagenesis using recombineering. J Biomed Biotechnol 2011:971296
78. Ciotta G, Hofemeister H, Maresca M, Fu J, Sarov M et al (2011) Recombineering BAC transgenes for protein tagging. Methods 53(2):113–119
79. Copeland NG, Jenkins NA, Court DL (2001) Recombineering: a powerful new tool for mouse functional genomics. Nat Rev Genet 2(10):769–779
80. Liu Q, Segal DJ, Ghiara JB, Barbas CF 3rd (1997) Design of polydactyl zinc-finger proteins for unique addressing within complex genomes. Proc Natl Acad Sci U S A 94(11):5525–5530
81. Boch J, Scholze H, Schornack S, Landgraf A, Hahn S et al (2009) Breaking the code of DNA binding specificity of TAL-type III effectors. Science 326:1509–1512
82. Moscou MJ, Bogdanove AJ (2009) A simple cipher governs DNA recognition by TAL effectors. Science 326:1501
83. Sun N, Abil Z, Zhao H (2012) Recent advances in targeted genome engineering in mammalian systems. Biotechnol J 7(9):1074–1087
84. Carroll D (2011) Genome engineering with zinc-finger nucleases. Genetics 188(4):773–782. doi:10.1534/genetics.111.131433, Review
85. Sun N, Zhao H (2013) Transcription activator-like effector nucleases (TALENs): a highly efficient and versatile tool for genome editing. Biotechnol Bioeng 110(7):1811–1821
86. Wang H, Hu Y-C, Markoulaki S, Welstead GG, Cheng AW et al (2013) TALENmediated editing of the mouse Y chromosome. Nat Biotechnol 31:530–532
87. Hsu PD, Lander ES, Zhang F (2014) Development and applications of CRISPR-Cas9 for genome engineering. Cell 157:1262–1278
88. Sander JD, Joung JK (2014) CRISPR-Cas systems for editing, regulating and targeting genomes. Nat Biotechnol 32(4):347–355. doi:10.1038/nbt.2842, Epub 2014 Mar 2
89. Mali P, Esvelt KM, Church GM (2013) Cas9 as a versatile tool for engineering biology. Nat Methods 10(10):957–963. doi:10.1038/nmeth.2649, Review
90. Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW et al (2013) One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell 153:910–918
91. Auer TO, Duroure K, De Cian A, Concordet JP, Del Bene F (2014) Highly efficient CRISPR/Cas9-mediated knock-in in zebrafish by homology-independent DNA repair. Genome Res 24(1):142–153
92. Lee HJ, Kweon J, Kim E, Kim S, Kim JS (2012) Targeted chromosomal duplications and inversions in the human genome using zinc finger nucleases. Genome Res 22(3):539–548
93. Piganeau M, Ghezraoui H, De Cian A, Guittat L, Tomishima M et al (2013) Cancer translocations in human cells induced by zinc finger and TALE nucleases. Genome Res 23(7):1182–1193
94. Choi PS, Meyerson M (2014) Targeted genomic rearrangements using CRISPR/Cas technology. Nat Commun 5:3728
95. Torres R, Martin MC, Garcia A, Cigudosa JC, Ramirez JC et al (2014) Engineering human tumour-associated chromosomal translocations with the RNA-guided CRISPR-Cas9 system. Nat Commun 5:3964

Chapter 21
Protein Complex Hierarchy and Translocation Gene Products

Jacqueline M. Matthews

Contents

21.1 Proteins Function Within Networks of Interactions 448
21.2 LMO2 and Transcriptional Programmes in T-ALL 451
21.2.1 Translocations Involving LMO2 and T-ALL 451
21.2.2 The Structure and Function of LMO2 451
21.2.3 Molecular Targeting of LMO2 453
21.3 MYC and Transcriptional Regulation in Burkitt Lymphoma 453
21.3.1 MYC and Burkitt Lymphoma 453
21.3.2 The Structure and Function of MYC 454
21.3.3 Additional Factors in Oncogenic Transformation by MYC 455
21.3.4 Molecular Targeting of MYC 456
21.4 The Philadelphia Chromosome, Signalling and Cell Cycle Regulation 456
21.4.1 The Philadelphia Chromosome and CML 456
21.4.2 The Structure and Function of ABL1 456
21.4.3 The Structure and Function of BCR 457
21.4.4 BCR-ABL1 Fusion Proteins 458
21.4.5 Molecular Targeting of BCR-ABL1 459
21.5 BCL2 and the Inhibition of Apoptosis 459
21.5.1 BCL2 and Follicular Lymphoma 459
21.5.2 The Structure and Relationships of BCL2 Family Proteins 459
21.5.3 The Role of BCL2-Family Proteins in Apoptosis 460
21.5.4 Molecular Targeting of BCL2 462
21.5.5 Conclusion 462
References 462

Abstract Disease-causing chromosomal translocations tend to cause the up-regulated expression of proteins, or result in fusion proteins with altered functionality. Four sets of chromosomal translocations are presented as case studies to illustrate how the protein products of chromosomal translocations disrupt normal cellular processes through a range of different mechanisms. For translocations affecting *LMO2* and *MYC* expression, alterations to transcriptional regulation ultimately cause disease. In the case of the Philadelphia Chromosome, BCR-ABL1

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disrupts cell signalling and cell cycle regulation by generating an always active form of the ABL1 tyrosine kinase. Upregulation of *BCL2* blocks apoptosis. In each case the molecular basis of activity, and strategies for inhibition by directly targeting the disease causing proteins are summarized.

Keywords Apoptosis • Kinases • Fusion proteins • Protein over-expression • Transcriptional regulation

21.1 Proteins Function Within Networks of Interactions

In biology, molecules rarely, if ever, exist in isolation. Rather it is the dynamic interplay between different entities, whether they be protein, nucleic acids, lipids, carbohydrates or small molecules, that mediate biological processes. During normal development and homeostasis, these interactions are regulated, at least in part, through the availability of the different components. Alterations of normal cellular levels of proteins and other components can cause major disruptions to these networks of biomolecular interactions.

Disease-causing chromosomal translocations tend to cause the up-regulation of proteins such that they are expressed in cells where they wouldn't normally be present, are expressed at much higher levels than is normal, or have been otherwise activated as a result of the translocation. There are several different types of molecular outputs. In some cases fusion proteins are produced via the splicing of two different protein-coding regions of genes resulting in new functionality through the generation of multifunctional polypeptides. In other cases, fusion events cause regulatory parts of a gene (e.g., promoters or enhancers) to influence the protein coding region of a second gene, resulting in the up-regulated protein expression of an otherwise wild-type protein. In either case, cell-specific promoters often drive the overexpression of proteins or parts of proteins that shouldn't be in those cells, or would be present only at much lower levels. For example, promoters from T-cell receptor genes in T-cells, or immunoglobulin genes in B-cells, can drive T-cell and B-cell malignancies, respectively. Gene fusion events can also result in the loss of regulatory regions of proteins so that mechanisms that dampen down gene transcription or translation in that cell type are no longer in force.

Many different types of regulatory proteins are associated with chromosomal translocations. A small number of these are shown in Table 21.1 and include large numbers of transcription factors/transcriptional regulators and kinases, but also feature tumour suppressors, growth factors and RNA-binding proteins. Four examples are described in more detail in the remainder of this chapter.

Table 21.1 Selected examples of chromosomal translocations, their associated diseases and protein products

Translocation	Associated disease(s)	First gene	Second gene	Protein product
t(8;14)(q24;q32)[a]	Burkitt's lymphoma	*MYC* (Transcription factor)	*IGH@* (immunoglobulin heavy locus)	Overexpression of MYC
t(11;14)(q13;q32) [70]	Mantle cell lymphoma	*CCND1/Cyclin D1* Cell cycle regulation	*IGH@* (immunoglobulin heavy locus)	Overexpression of Cyclin D1
t(14;18)(q32;q21)[b]	Follicular lymphoma	IGH@ (immunoglobulin heavy locus)	*BCL2*	Overexpression of BCL2
t(10;(various)) (q11;(various)) [71]	Papillary thyroid cancer	*RET* (cadherin superfamily receptor tyrosine kinase)	Various	Constitutive activation of RET tyrosine-kinase
t(2;3)(q13;p25) [72]	follicular thyroid cancer	*PAX8* (homeodomain transcription factor)	*PPARPG/PPARγ1* (peroxisome proliferator-activated nuclear receptor)	PAX8-PPARG fusion protein (transcriptional activity)
t(8;21)(q22;q22) [73]	AML	*CBFA2T2/ETO* (transcriptional regulator)	*RUNX1/AML1* (transcription factor)	AML1-ETO fusion protein (transcriptional activity)
t(9;22)(q34;q11)[c] Philadelphia chromosome	CML, ALL	*ABL1* (non receptor tyrosine kinase)	*BCR* (GTPase-activating protein serine/threonine kinase)	BCR-ABL fusion protein
t(15;17)(q22;q21) [74]	Acute promyelocytic leukaemia	*PML* (TRIM-family protein/ transcription factor)	*RARA/RAR-α* (nuclear retinoic acid receptor/transcriptional regulation)	PML-RAR fusion protein (self-association of RAR-α)
t(11;19)(q21; p12~p13.11)	Pulmonary mucoepidermoid carcinoma	*MAML2* (transcriptional activator of Notch proteins)	*CRTC1/MECT1* (CREB-dependent transcriptional coactivator)	MECT1-MAML2 fusion protein (disrupted cAMP/CREB pathways)

(continued)

Table 21.1 (continued)

Translocation	Associated disease(s)	First gene	Second gene	Protein product
t(15;19)(q14;p13.1) [75]	Midline carcinoma	*NUTM1*	*BRD4* (bromodomain-containing protein)	BRD4-NUTM1 fusion protein (deregulation of histone acetyltransferase activity)
t(11;22)(q24;q11.2-12) [76]	Ewing's sarcoma	*FLI1/ERGB* (Transcription factor)	*EWSR1(*EWS RNA-binding protein)	EWS/FLI (transcriptional down-regulation)
t(X;18)(p11.2;q11.2) [77]	Synovial sarcoma	*SSX1* or *SSX2* Transcriptional repressors	*SYT* (Integral membrane proteins of synaptic vesicles)	SYT-SSX1/2 fusion proteins (localisation of SSX1/2 to nuclear bodies)
t(17;19)(q22;p13) [78]	B-ALL	*HLF* (PAR subfamily of basic leucine zipper)	*TCF3/E2A* Common bHLH transcription factor	E2A-HLF (N-terminus of E2A fused to bZIP from HLF)
t(12;15)(p12;q26.1) [79]	Secretory breast carcinoma/congenital fibrosarcoma	*ETV6* (ETS family transcription factor)	*NTRK3* (neurotrophic tyrosine receptor kinase)	ETV6-NTRK3 fusion protein (bHLH dimerization domain of ETV6 fused to the protein tyrosine kinase (PTK) domain of NTRK3)
t(12;15)(p13;q25) [80]				
t(11;14)(p13;q11) t(7;11)(q35;p13)[d]	T-ALL	*TRD@*) or *TRB@* (T-cell receptor gene loci)	*LMO2* (transcriptional regulator)	Overexpression of LMO2 in T-cells

[a] See Sect. 21.3
[b] See Sect. 21.5
[c] See Sect. 21.4
[d] See Sect. 21.2

21.2 LMO2 and Transcriptional Programmes in T-ALL

21.2.1 Translocations Involving LMO2 and T-ALL

LIM-only protein 2 (LMO2/Rhombotin 2) was originally discovered in association with chromosomal translocations in acute lymphoblastic T-cell leukaemia (T-ALL) in children. LMO2 is a transcriptional regulator that has important roles in several stages of blood cell development, including the maintenance of stem cells and red blood cell development. Although LMO2 is present at the early stages of T-cell development, under normal circumstances its expression is down-regulated at the DN2 stage of development [12]. In mouse models, and in gene therapy trials in humans (where retroviral insertion of occurred close to *LMO2*), forced expression of LMO2 in T-cells leads to the onset of T-ALL with a long onset period. For both chromosomal translocations that lead to T-ALL, t(11;14)(p13;q11.2) and t(7;11)(q34;p13), T-cell receptor genes (the δ-chain gene (*TRD*) at 14q11.2 and the β-chain gene (*TRB*) at 7q34) are fused upstream of *LMO2* at 11p13, leading to high levels of LMO2 in T-cells [13–15]. However, in mice that carry non-conditional transgenes for *LMO2* (i.e., the transgene should be expressed in all cells), the only observed phenotype is T-ALL with a long period of onset [16, 17]. In addition to chromosomal translocations, a cryptic deletion (del(11)(p12p13)) around *LMO2* leads to T-ALL. The deletion is postulated to remove a negative regulatory element leading to increased expression of the protein [18].

21.2.2 The Structure and Function of LMO2

The sequence of LMO2 contains little more than two LIM domains that mediate interactions with other proteins (e.g., Fig. 21.1a). Thus, LMO2 functions by taking part in transcriptional complexes. Recent gene expression and multi-factorial chromatin immunoprecipitation with deep sequencing studies indicate that LMO2 is one of a set of transcription factors and regulators (including LMO1/2, LDB1, CBFA2T3 (also known as ETO2), GATA1/GATA2/GATA3, TAL1/LYL1, RUNX1 family and ETS family proteins) that act in a combinatorial fashion to regulate gene expression a range of blood cell types. The indications are that LMO2 can take part in a variety of transcriptional complexes, but its best characterized role is as a bridging factor between the DNA-binding basic helix-loop-helix and GATA proteins at bipartite E-box/GATA sites, and the cofactor protein LDB1 (LIM domain binding protein 1), which mediates long range chromatin interactions.

LMO2 is required for haematopoietic stem cell (HSC) maintenance [20]. The upregulation of LMO2 in T-cells disrupts transcriptional programmes such that T-cell differentiation genes are down-regulated (e.g., by sequestration of *TFPT*/*E2A* genes into LMO2-containing transcriptional complexes) and HSC genes are upregulated [21] (Fig. 21.1b). Although other mechanisms may contribute [22, 23], the

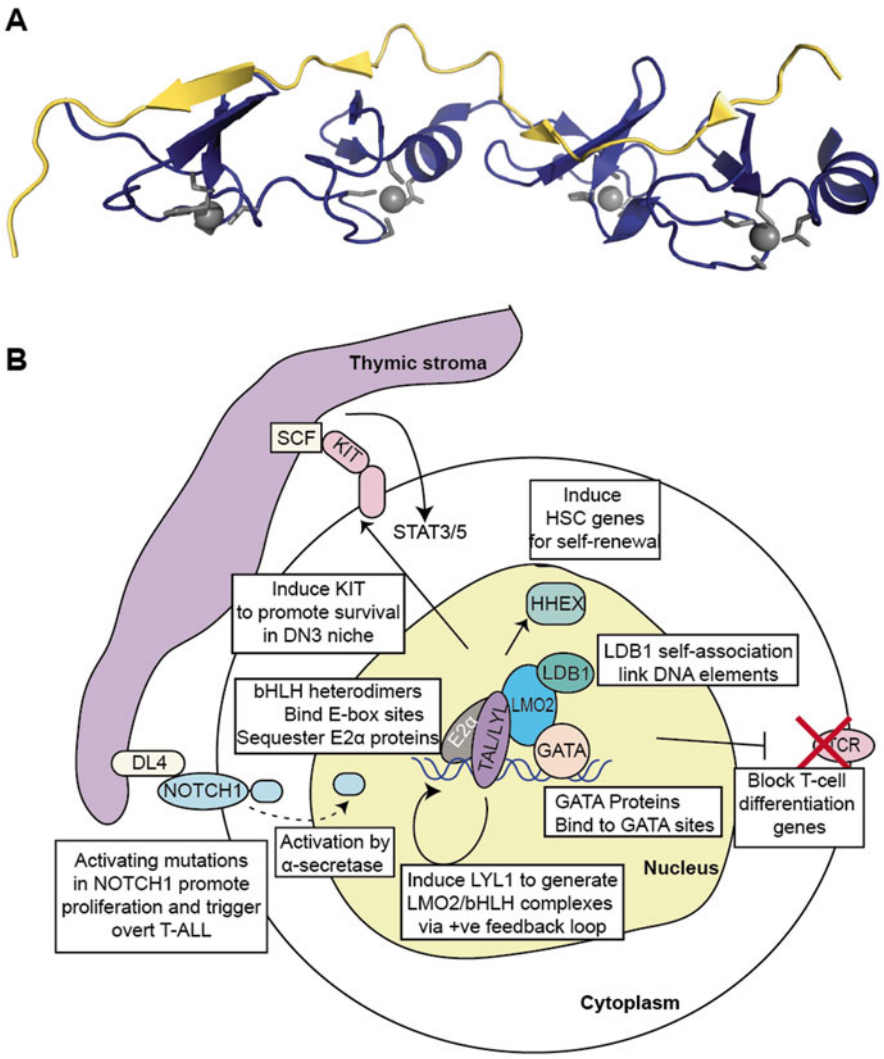

Fig. 21.1 LMO2 and T-ALL. (**a**) Structure of LMO2 (*Blue*) bound to LDB1 (*Yellow*; Pdb code: 2XJY). Zinc atoms shown as *grey spheres* and zinc-coordinating side-chains as *grey sticks*. (**b**) Mechanisms of T-ALL induction by LMO2 (Originally published in a copy edited form in Ref. [19])

result is the introduction of stemness to pre-leukaemia DN3 cells (for example by activating the *NKL* homeobox genes, *HHEX*) allowing clonal expansion and the opportunity to acquire the transformative mutations that lead to T-ALL.

21.2.3 Molecular Targeting of LMO2

To date, Rabbitts and colleagues have developed intracellular antibodies [24, 25] and peptides [26] that have anti-LMO2 activity in both cell-based erythropoiesis assays and tumourigenesis using a mouse explant model of LMO2-induced leukaemia. A single-variable domain intracellular antibody binds to an extended binding surface on LMO2 [27], whereas the CXXC-containing peptides likely destabilize LMO2 through disruptions to zinc-coordination [28].

21.3 MYC and Transcriptional Regulation in Burkitt Lymphoma

21.3.1 MYC and Burkitt Lymphoma

A defining feature of Burkitt lymphoma (a highly aggressive B-cell malignancy first described by Burkitt in 1958 in equatorial Africa) is the presence of a chromosomal translocation between *MYC* (*v-myc* Avian Myelocytomatosis Viral Oncogene Homolog/c-*myc*) from chromosome 8 and immunoglobulin genes. In the majority of cases (80–90 %) this involves the immunoglobulin heavy chain gene (IGH, t(8;14)(q24.2;q32.3)), with the remainder involving the kappa (IGK, t(2;8)(p12;q24.2)) or lambda light chain genes (IGL), t(8;22)(q24.2;q11.2)). The same translocations are found in some cases of diffuse large B-cell lymphomas (DLBCL), follicular lymphoma and mantle cell lymphoma, where they correlate with a more aggressive phenotype and lower survival rate [29]. In these cases the chromosomal translocations are associated with higher age and cytogenetic complexity pointing to the translocation being a secondary event rather than the initial cause of oncogenic transformation [29]. Burkitt lymphoma is subdivided into three epidemiologically distinct forms: endemic (common to equatorial Africa and New Guinea, and usually associated with Epstein Barr virus), sporadic (1–2 % of adult lymphomas in Europe and the USA) and HIV-associated.

21.3.2 The Structure and Function of MYC

MYC belongs to the MYC family of transcriptional factors, which also includes MYCN/N-Myc, and MYCL/l-Myc. These proteins contain a basic-helix-loop-helix (bHLH)/Leucine zipper (LZ) domain that forms heterodimers with other bHLH/LZ transcription factors such as MAX, and binds to Enhancer (E)-box motifs on DNA (Fig. 21.2a, b). The N-terminus of MYC contains three so-called MYC-boxes that are essential for protein stability, protein interaction, and transcriptional activation or repression of MYC target genes [31]. The basic domain in the bHLH motif makes contacts with DNA, and the HLH/LZ and MYC-boxes mediate interactions with MAX and other proteins, respectively, that will modulate the DNA-binding specificity of MYC. MYC itself is a global transcriptional activator that has been described as acting primarily to modulate chromatin structure; however, other transcriptional regulators bind to, and act concert with, MYC in a cell type dependent manner such that MYC can act as a repressor or an activator of transcription depending on its binding partners [31, 32].

MYC is one of the four Yamanaka factors (also including KLF4, POU5F1/OCT4, SOXS2) that when combined are sufficient to reprogramme differentiated cells to

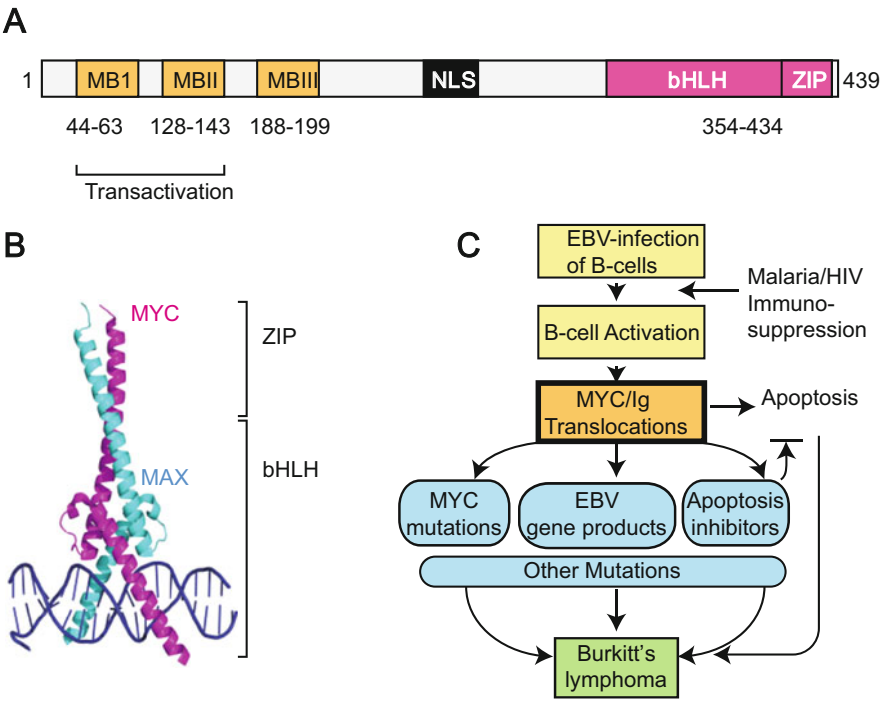

Fig. 21.2 MYC. (**a**) Domain structure of MYC (not to scale). (**b**) Structure of the MYC-MAX heterodimer bound to E-box DNA (PDB code – 1NKP). (**c**) Interplay of viruses and MYC/IG translocations in Burkitt lymphoma (Adapted from Fig. 21.1, Ref. [30])

become induced pluripotent stem cells [33]. The protein has been estimated to bind up to 15 % of human genes, including those involved in cell growth, cell cycle progression, apoptosis, differentiation, protein biosynthesis, and energy metabolism, adhesion and migration, angiogenesis, chromosomal stability and stem cell renewal [34]. MYC also controls complex networks of microRNAs, as least in part through transcriptional regulation of the miRNA machinery [35].

21.3.3 Additional Factors in Oncogenic Transformation by MYC

Whereas Burkitt lymphoma is relatively rare, MYC is overexpressed in up to 70 % of human cancers. Mutations have also been reported that increase expression, activity and stability of MYC (e.g., by preventing proteolytic degradation of the protein [36]). These mutations likely occur after the chromosomal translocation. In general, the upregulation of *MYC* appears to upset the balance between apoptosis and proliferation. However, upregulation of *MYC* is not sufficient to trigger oncogenic transformation. Indeed, in most cells the overexpression of *MYC* triggers apoptosis, which acts in direct opposition to clonal expansion. Additional changes must accumulate in affected cells that counteract MYC-induced apoptosis (e.g., Fig. 21.2c). Numerous examples of such changes have been reported, including inactivating mutations in the TP53/CDK2NA/MDM2 pathway, down-regulation of pro-apoptotic proteins or up-regulation of anti-apoptotic proteins [37]. For example, 'double hit' lymphomas/leukaemias that carry translocations involving *MYC* and *BCL2* (see below) or three-way translocations involving *MYC*, *BCL2* and immunoglobulin genes are particularly aggressive (e.g., [38]).

With so many different targets, the oncogenic transformation of cells by MYC, whether stemming from translocations or other modes of up-regulation, involve multiple different pathways (e.g., [39–41]). Interaction with viruses appears to play a prominent role in endemic and HIV-associated Burkitt lymphoma. EBV was first isolated from endemic Burkitt lymphoma patients and is detected in close to 100 % of these patients. However, despite the longstanding epidemiological links, the molecular basis of oncogenic transformation by EBV remains elusive [42]. Although most people carry apparently harmless latent infections by EBV, only ~5–10 % of sporadic cases show evidence of association with EBV [30]. It is generally considered that EBV is present prior to the chromosomal translocation, and subsequent or co-infection by malaria or HIV induces immunosuppression and provokes expansion of germinal centre or memory B-cells leading to chromosomal translocations [30] (Fig. 21.2c). EBV appears to provide protection against MYC-induced apoptosis by expression of the EBV proteins EBNA-1 and EBERs, some of which may act, at least in part, by down-regulating the expression of cellular pro-apoptotic proteins (e.g. reviewed in [30]). About 40 % of HIV-associated Burkitt lymphoma cases are also EBV-associated.

21.3.4 Molecular Targeting of MYC

In its monomeric form, MYC is intrinsically disordered. The flexibility of intrinsically disordered proteins presents a challenge for rational inhibitor design. However, small molecules have been identified that bind to MYC [43, 44]. 10058-F4 and 10074-G5 stabilize the intrinsically disordered monomer over the highly ordered MYC-MAX heterodimer through binding to multiple distinct regions of MYC [45]. Although the current generation of inhibitors have limited efficacy in vivo, apparently due to poor bioavailability and/or rapid metabolic breakdown [46], this approach of inhibiting the transcriptional activity of MYC shows some promise (reviewed in [47]).

21.4 The Philadelphia Chromosome, Signalling and Cell Cycle Regulation

21.4.1 The Philadelphia Chromosome and CML

The Philadelphia chromosome (Ph), t(9;22)(q34.1;q11.2), is one of the earliest and best known chromosomal translocations to be associated with malignancy. It is a characteristic of chronic myeloid leukaemia (CML; 95 % of CML patients carry this translocation), is found in acute lymphoblastic leukaemia (ALL; the Philadelphia chromosome is associated with up to 30 % of adult and 10 % of childhood cases of ALL) and more rarely, in acute myeloid leukaemia (AML). The translocation involves the fusion of the breakpoint cluster region (*BCR*) gene on chromosome 22 with *ABL1* (the human homologue of the Abelson murine leukaemia virus) oncogene on chromosome 9.

21.4.2 The Structure and Function of ABL1

abl was discovered in 1980 as the oncogene in the Abelson murine leukaemia virus [48]. It encodes a large non-receptor tyrosine kinase that contains multiple protein-interaction domains including an SH2 domain (for interaction with phorphorylated proteins), an SH3 domain (for interaction with proline-rich proteins), a proline-rich region, (for interaction with SH3-domain-containing proteins) and actin binding domains, as well as DNA-binding domains and nuclear localisation and export sequences (Fig. 21.3a). ABL1 interacts with a large variety of cellular proteins, including signalling adaptors, kinases, phosphatases, cell-cycle regulators, transcription factors and cytoskeletal proteins. It is thought to function in a wide range of cellular processes, including regulation of cell growth and survival, oxidative stress and DNA-damage responses, actin dynamics and cell migration [49]. The wild-type protein is alternatively spliced at the N-terminus such that one isoform can be myristylated, whereas the other is not. The kinase activity of ABL1 is tightly regulated by

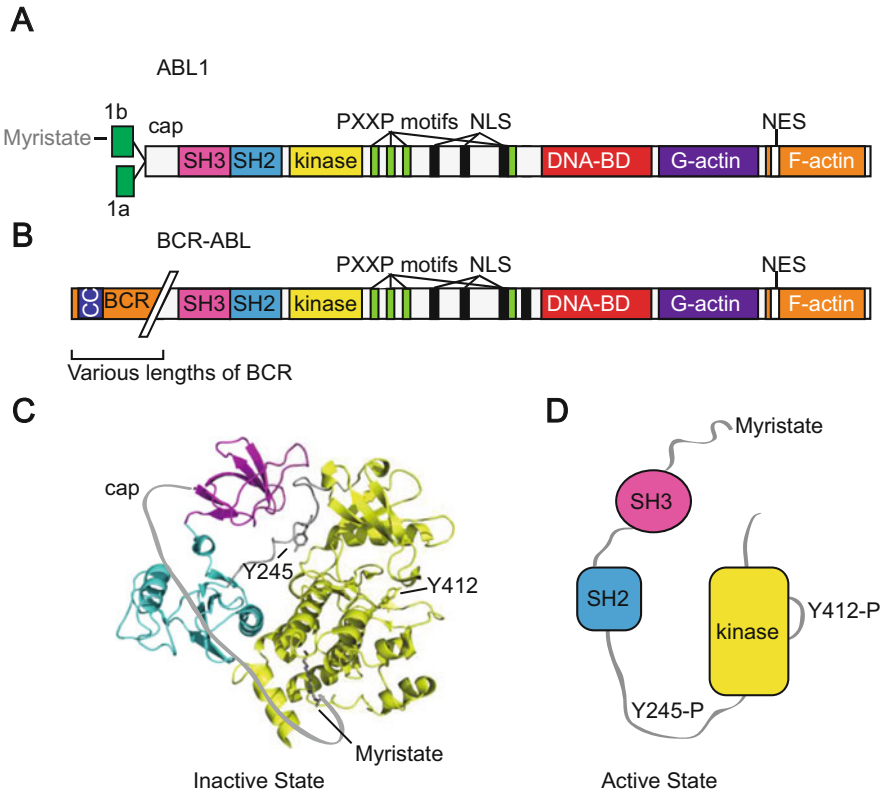

Fig. 21.3 BCR-ABL1. Domain structure of (**a**) ABL1 and (**b**) BCL-ABL1 (not to scale). (**c**) Structure of the inactivate state of ABL1 (N-terminal to kinase domains only; PDB code-1OPK). (**d**) Schematic of the active state (same regions). Residues Y245 and Y412, and the N-terminal myristate stabilize the inactive state by forming interdomain interactions. Phosphorylated Y245 and Y412 cannot make the same interactions, and loss of the N-terminal myristate in BCR-ABL1 promotes an 'always activated' state. The coiled-coil domain in BCR is also thought to be involved in activating the ABL1-kinase domain in BCR-ABL1

both intermolecular and intramolecular interactions, and by post-translational modifications. For example, autoinhibition is achieved through a complex series of interdomain interactions and associated conformational changes that involves contacts between the myristylated N-terminus and kinase domain [50] (Fig. 21.3b).

21.4.3 The Structure and Function of BCR

BCR is an ~140-kDa protein whose function is still largely unknown. Whereas mice carrying a *BCR-ABL1* transgene develop CML [51], mice that are homozygous null for *BCR* have defects in hormonal and behavioural stress response

regulation and are prone to septic shock (MGI database). The protein has serine/threonine kinase activity [52] and is a GTPase-activating protein for RAC1 and RAC2 and CDC42 [53, 54], and is reported to bind a range of other proteins. BCR also contains an N-terminal tetramerisation domain that forms a helical bundle [55].

21.4.4 BCR-ABL1 Fusion Proteins

The protein products of the Philadelphia chromosome vary in size according to the breakpoints in the translocation. Although different breakpoint sequences of ABL1 are involved, splicing of the primary hybrid transcript invariably yields an mRNA molecule in which *BCR* sequences are fused to *ABL1* exon a2. Thus, all but the N-terminal region of ABL1 is expressed (Fig. 21.3a). The variation in protein size stems from the BCR component, such that three different fusion proteins are generated, which tend to be associated with distinct leukaemia types. A 210 kDa protein is a hallmark of CML, a 185 kDa protein is present in 20–30 % of adult and in 3–5 % of childhood B-ALL [56, 57], and a 230-kDa protein has been found in chronic neutrophilic leukaemia (and more rarely in CML) [58].

All of these BCR-ABL1 proteins result in constitutively activated ABL1 tyrosine kinase activity. This is brought about, at least in part, by loss of the myristylated N-terminus of ABL1, thereby preventing autoinhibition, but also by the presence of regions from BCR. For example, the BCR N-terminal oligomerization domain and SH2-binding elements (that can target the SH2 domain from ABL1) are required for oncogenic transformation [59–61]. The correlation of different fusion proteins with distinct leukaemias suggests that other portions of BCR affect the function of the fusions and the phenotype of affected cells. Although the BCR regions contain serine/threonine kinase activity, it is the tyrosine kinase activity, and the ability of ABL1 to modify a large number of targets in cells, that is generally thought to trigger disease. Indeed, *ABL1* has been identified as a fusion partner with at least eight other fusion partners in rare chromosome translocations associated with T-ALL, B-ALL and other leukaemias and ABL1 has an emerging role activated in various solid tumours [62]. Thus, the tyrosine kinase activity of ABL1 (see below) has been a very effective target for drug therapies against CML and other cancers.

Multiple signalling pathways appear to mediate the oncogenic activity of BCR-ABL1, including the RAS, nuclear factor-κB (NFKB1), PIK3CA/PI3K-AKT1, JUN, CTNNB1 and signal transducer and activator of transcription 3 (STAT3) signalling pathways (e.g., [62, 63]). It should be noted, however, that the Philadelphia chromosome is not itself sufficient to cause leukaemia; low levels of *BCR-ABL1* fusion transcripts have been detected in up to 75 % of healthy individuals [64].

21.4.5 Molecular Targeting of BCR-ABL1

Small molecules that inhibit BCR-ABL1 generally target the tyrosine kinase activity of ABL1. CML was essentially a fatal disorder until Imatinib/Gleevec/STI571 [65] was developed to target the ATP-binding site of BCR-ABL1. This was the first targeted therapy for cancer treatment, being specific for the ABL1 tyrosine kinase but not other similar kinases. Although the drug revolutionized treatment of CML, some patients cannot tolerate it, others develop resistance, and relapse can occur if the malignant clone is not eradicated, leading to the development of subsequent generations of inhibitors that give improved survival rates for CML patients (reviewed in [66]).

21.5 BCL2 and the Inhibition of Apoptosis

21.5.1 BCL2 and Follicular Lymphoma

BCL2 was discovered in association with the t(14;18)(q32.3;q21.3) chromosomal translocation in which the *BCL2* gene from chromosome 18, is juxtaposed with the *IGH* gene on chromosome 14 [67–69]. This translocation is characteristic of follicular lymphoma, with an occurrence rate of 80–90 %. The same translocation is seen in ~20–30 % of cases of de novo DLBCL and rare cases of chronic lymphocytic leukaemia (CLL) [70]. Variant chromosomal translocations involving *BCL2* and other partner genes have been described that are not preferentially associated with follicular lymphoma. These include the juxtaposition of BCL2 with either the immunoglobulin (IG) κ or λ genes, located at chromosome 2p12 [71] or 22q11.2 [72], respectively. With the use of conventional cytogenetics methods, about 20 lymphomas or leukaemias with variant translocations involving *BCL2* have been reported in the literature. One study that used Southern blot analysis and restriction fragment mapping identified *BCL2* gene rearrangement with an IG light chain gene in 3 (9.4 %) of 32 CLL cases [73].

21.5.2 The Structure and Relationships of BCL2 Family Proteins

As the name suggests, BCL2 was the first discovered of the BCL2 family proteins, which also includes the other anti-apoptotic/pro-survival proteins (BCL2L1/BCL-XL, BCL-W, A1/BCL2A1, MCL1), the pro-apoptotic proteins (BAX, BAK1, and BOK) and the less-well studied members whose designations are less well established (BCL-RAMBO/BCL2L13, BOO/BCL2L10, and BCLG/BCL2L14). Despite significant sequence variation, these proteins share up to four regions of

conserved sequence known as BCL2 homology (BH) domains, BH1–BH4 (Fig. 21.4a). These multidomain BCL2 family proteins form a common fold in which seven amphipathic helices pack against a central solvent inaccessible hydrophobic helix (Fig. 21.4b). A hydrophobic groove formed on the surface of the proteins can bind helical BH3 domains from BH3-only proteins (BCL2L11/BIM, BID, BBC3/PUMA, BAD, HRK, BIK, BCL2L15/BFK and PMAIP1/NOXA; e.g. Fig. 21.4c) or small molecule inhibitors (Fig. 21.4d). However, there is some selectivity in binding. For example, BCL2L11/BIM, BID and BBC3/PUMA each bind to all five pro-survival BCL2 family members, whereas BAD and PMAIP1/NOXA bind subsets of these proteins (BCL2, BCL2L1/BCL-XL, BCL-W) and (AI, MCL10), respectively. Many of these proteins also contain a putative transmembrane region and are typically associated with membranes. For example, BCL2 was detected in the nuclear outer membrane, mitochondrial outer (and to a lesser extent the inner) membranes and the endoplasmic reticulum [75].

21.5.3 The Role of BCL2-Family Proteins in Apoptosis

BCL2 contributes to carcinogenesis by inhibiting cell death [76]. The BCL2 family of proteins predominantly regulate the initiation of apoptosis by the intrinsic or mitochondrial pathway (Fig. 21.4e). Diverse stimuli (such as growth factor deprivation, stress, UV radiation or viruses) typically activate one or more BH3-only proteins. Pro-survival proteins, including BCL2, bind to and inhibit these proteins. However, once a threshold of BH3-activation is reached, this inhibition is overcome, leading to the assembly of BAK or BAX oligomers within the outer mitochondrial membrane. These oligomers permeablize the mitochondria, by as yet undetermined mechanisms, leading to cytochrome C release, which in turn triggers apoptosome assembly and the cascades of caspsase activation that cause demolition of the cell (e.g., reviewed in [77]). The BCL2 proteins also provide some links between the intrinsic and extrinsic apoptotic pathways, through caspase-8 activation of BID in the latter pathway. Activated BID (tBID) then enters the intrinsic pathway.

The interplay between the pro-survival and pro-apoptotic BCL2 proteins relies heavily on the insertion of BH3-domains (from both multidomain and BH3-only proteins) into the hydrophobic groove on the multidomain proteins to either inhibit or activate mitochondrial permeabilization. In healthy cells, the pro-apoptotic BAX is monomeric, inert and largely restricted to the cytoplasm. During apoptosis, it becomes translocated to the outer mitochondrial membrane. Activation of BAX by pro-apoptotic BH3 domains triggers a conformational change that generates a metastable state before ultimately resulting in BAX oligomers [78]. BCL2 can inhibit BAX activation by sequestering pro-apoptotic BH3 domains, but BCL2 may also directly prevent BAX oligomerization through interaction of the two proteins in the outer mitochondrial membrane, which has been reported to require conformational changes in both BCL2 and BAX [79]. In addition to binding to other BCL2 family proteins, BCL2 can reportedly bind proteins involved in other aspects of apoptosis

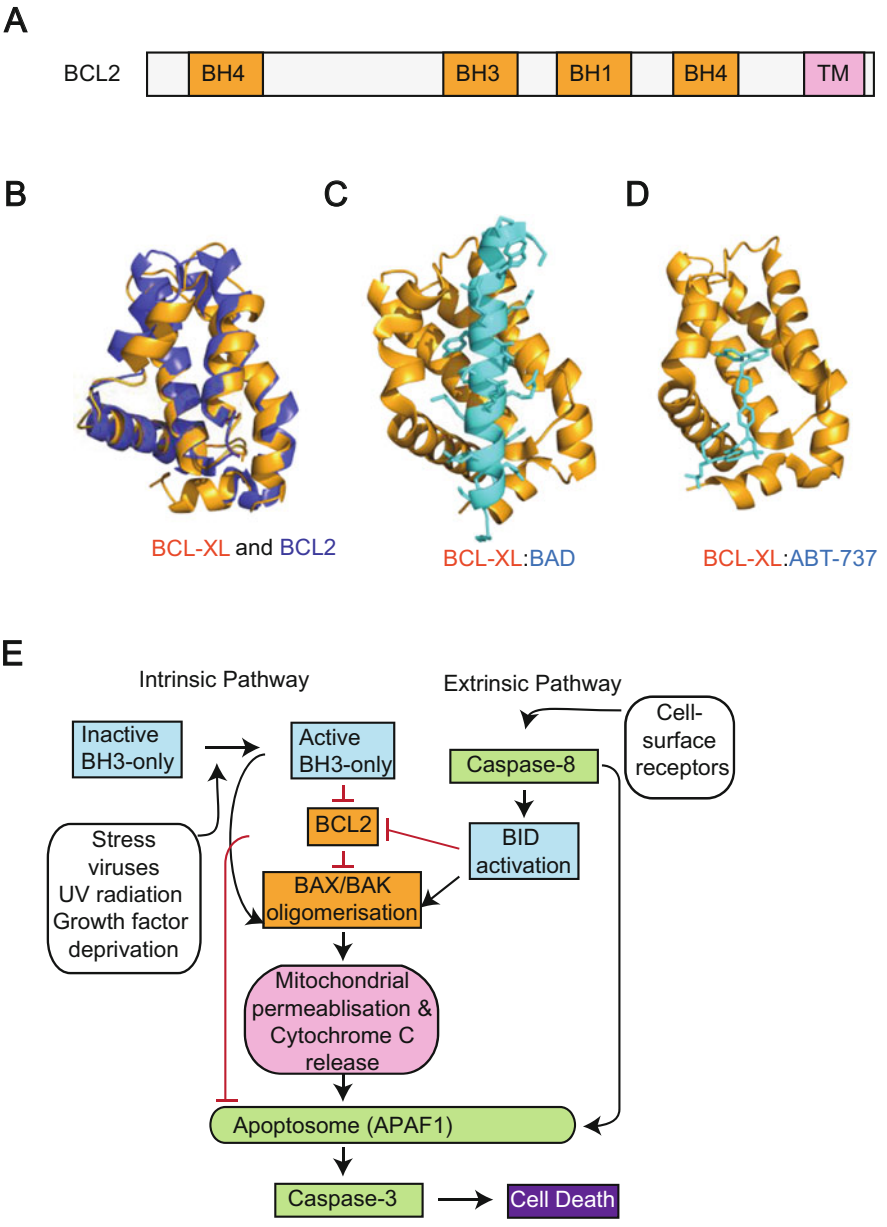

Fig. 21.4 BCL2. (**a**) Domains in BCL2 (BCL2-homology domains, BH1–4; Transmembrane domain, TM; not to scale). (**b**) Overlay of the structures of BCL2 (PBD code – 1G5M; *Blue*) and BCL2L1/BCL-XL (PDB code – 1LXL; *orange*). (**c**) Structure of BCL2L1/BCL-XL (*orange*) bound to the BH3-only protein BAD (*cyan*; PDB code – 2BZW). (**d**) Structure of BCL2L1/BCL-XL (*orange*) bound ABT-737(*cyan*; PDB code – 2YXJ). (**e**) Roles of BCL2 in apoptosis. *Black arrows* indicate pro-apoptosis pathways, *red bars* indicate inhibition of apoptosis (Adapted from Fig. 21.2, Ref. [74])

(such as APAF1 and related proteins), as well as other cellular processes. Notably, the ability of BCL2 to inhibit apoptosis is probably not sufficient to drive oncogenic transformation; the concomitant stimulation of cell proliferation and inhibition of apoptosis are both required [80]. Thus, the cooperation between the anti-apoptotic activity of BCL2 and pro-proliferation properties of proteins, such as MYC (as described above for MYC) or other changes drive oncogenesis. BCL2 does localize to nuclear membranes and some reports indicate that BCL2 can inhibit DNA repair, which could contribute to genome instability in cells that overexpress BCL2 and thereby lead to secondary mutations that cause oncogenic transformation [81].

21.5.4 Molecular Targeting of BCL2

BCL2-family proteins are a very active area for small molecule inhibitor development for use as therapeutics in cancer treatment. These BH3 mimetics include natural product derivatives and rationally designed compounds such as ABT-737 and its oral analogue ABT-263 (navitoclax), ABT-199, GX15-070 (obatoclax), R-(-)-gossypol (AT-101), WEHI-539, and BM series compounds (reviewed in [82]). Although many of these molecules display some levels of cytotoxicity, they do have anti-tumour properties derived from targeting one or more BCL2 family members.

21.5.5 Conclusion

These examples comprise a very small subset of known disease-associated chromosomal translocations (see other chapters for more details), but represent common modes of oncogenic transformation that occur as a result of translocations. Transcriptional programming, altered signalling pathways and blocks to apoptosis (often in combination) are the frequently observed outcomes from perturbations to normal cellular interaction networks. However, in many cases, inhibition of the aberrant interactions is an effective strategy for therapeutic treatment.

References

1. Jadayel DM, Lukas J, Nacheva E, Bartkova J, Stranks G, De Schouwer PJ et al (1997) Potential role for concurrent abnormalities of the cyclin D1, p16CDKN2 and p15CDKN2B genes in certain B cell non-Hodgkin's lymphomas. Functional studies in a cell line (Granta 519). Leukemia 11(1):64–72
2. Romei C, Elisei R (2012) RET/PTC translocations and clinico-pathological features in human papillary thyroid carcinoma. Front Endocrinol 3:54

3. Kroll TG, Sarraf P, Pecciarini L, Chen CJ, Mueller E, Spiegelman BM et al (2000) PAX8-PPARgamma1 fusion oncogene in human thyroid carcinoma [corrected]. Science 289(5483):1357–1360
4. Erickson P, Gao J, Chang KS, Look T, Whisenant E, Raimondi S et al (1992) Identification of breakpoints in t(8;21) acute myelogenous leukemia and isolation of a fusion transcript, AML1/ETO, with similarity to Drosophila segmentation gene, runt. Blood 80(7):1825–1831
5. Schoch C, Haase D, Haferlach T, Freund M, Link H, Lengfelder E et al (1996) Incidence and implication of additional chromosome aberrations in acute promyelocytic leukaemia with translocation t(15;17)(q22;q21): a report on 50 patients. Br J Haematol 94(3):493–500
6. French CA (2012) Pathogenesis of NUT midline carcinoma. Annu Rev Pathol 7:247–265
7. Turc-Carel C, Aurias A, Mugneret F, Lizard S, Sidaner I, Volk C et al (1988) Chromosomes in Ewing's sarcoma. I. An evaluation of 85 cases of remarkable consistency of t(11;22)(q24;q12). Cancer Genet Cytogenet 32(2):229–238
8. Brett D, Whitehouse S, Antonson P, Shipley J, Cooper C, Goodwin G (1997) The SYT protein involved in the t(X;18) synovial sarcoma translocation is a transcriptional activator localised in nuclear bodies. Hum Mol Genet 6(9):1559–1564
9. Hunger SP, Ohyashiki K, Toyama K, Cleary ML (1992) Hlf, a novel hepatic bZIP protein, shows altered DNA-binding properties following fusion to E2A in t(17;19) acute lymphoblastic leukemia. Genes Dev 6(9):1608–1620
10. Tognon C, Knezevich SR, Huntsman D, Roskelley CD, Melnyk N, Mathers JA et al (2002) Expression of the ETV6-NTRK3 gene fusion as a primary event in human secretory breast carcinoma. Cancer Cell 2(5):367–376
11. Knezevich SR, McFadden DE, Tao W, Lim JF, Sorensen PH (1998) A novel ETV6-NTRK3 gene fusion in congenital fibrosarcoma. Nat Genet 18(2):184–187
12. Herblot S, Steff AM, Hugo P, Aplan PD, Hoang T (2000) SCL and LMO1 alter thymocyte differentiation: inhibition of E2A-HEB function and pre-T alpha chain expression. Nat Immunol 1(2):138–144
13. Boehm T, Foroni L, Kaneko Y, Perutz MF, Rabbitts TH (1991) The rhombotin family of cysteine-rich LIM-domain oncogenes: distinct members are involved in T-cell translocations to human chromosomes 11p15 and 11p13. Proc Natl Acad Sci U S A 88(10):4367–4371
14. Royer-Pokora B, Loos U, Ludwig WD (1991) TTG-2, a new gene encoding a cysteine-rich protein with the LIM motif, is overexpressed in acute T-cell leukaemia with the t(11;14)(p13;q11). Oncogene 6(10):1887–1893
15. Van Vlierberghe P, van Grotel M, Beverloo HB, Lee C, Helgason T, Buijs-Gladdines J et al (2006) The cryptic chromosomal deletion del(11)(p12p13) as a new activation mechanism of LMO2 in pediatric T-cell acute lymphoblastic leukemia. Blood 108(10):3520–3529
16. Larson RC, Fisch P, Larson TA, Lavenir I, Langford T, King G et al (1994) T cell tumours of disparate phenotype in mice transgenic for Rbtn-2. Oncogene 9(12):3675–3681
17. Neale GA, Rehg JE, Goorha RM (1997) Disruption of T-cell differentiation precedes T-cell tumor formation in LMO-2 (rhombotin-2) transgenic mice. Leukemia 11(Suppl 3(13)):289–290
18. Hammond SM, Crable SC, Anderson KP (2005) Negative regulatory elements are present in the human LMO2 oncogene and may contribute to its expression in leukemia. Leuk Res 29(1):89–97
19. Matthews JM, Lester KL, Joseph S, Curtis DJ (2013) LIM-domain-only proteins in cancer. Nat Rev Cancer 12(2):111–122
20. Yamada Y, Warren AJ, Dobson C, Forster A, Pannell R, Rabbitts TH (1998) The T cell leukemia LIM protein Lmo2 is necessary for adult mouse hematopoiesis. Proc Natl Acad Sci U S A 95(7):3890–3895
21. McCormack MP, Young LF, Vasudevan S, de Graaf CA, Codrington R, Rabbitts TH et al (2010) The Lmo2 oncogene initiates leukemia in mice by inducing thymocyte self-renewal. Science 327(5967):879–883

22. Martins VC, Ruggiero E, Schlenner SM, Madan V, Schmidt M, Fink PJ et al (2012) Thymus-autonomous T cell development in the absence of progenitor import. J Exp Med 209(8):1409–1417
23. Peaudecerf L, Lemos S, Galgano A, Krenn G, Vasseur F, Di Santo JP et al (2012) Thymocytes may persist and differentiate without any input from bone marrow progenitors. J Exp Med 209(8):1401–1408
24. Nam CH, Lobato MN, Appert A, Drynan LF, Tanaka T, Rabbitts TH (2008) An antibody inhibitor of the LMO2-protein complex blocks its normal and tumorigenic functions. Oncogene 27(36):4962–4968
25. Tanaka T, Rabbitts TH (2009) Selection of complementary single-variable domains for building monoclonal antibodies to native proteins. Nucleic Acids Res 37(5):e41. [Research Support, Non-U.S. Gov't]
26. Appert A, Nam C-H, Lobato N, Priego E, Miguel RN, Blundell T et al (2009) Targeting LMO2 with a peptide aptamer establishes a necessary function in overt T-cell neoplasia. Cancer Res 69(11):4784–4790
27. Sewell H, Tanaka T, El Omari K, Mancini EJ, Cruz A, Fernandez-Fuentes N et al (2014) Conformational flexibility of the oncogenic protein LMO2 primes the formation of the multi-protein transcription complex. Sci Rep 4:3643
28. Wilkinson-White L, Matthews JM (2014) The PA207 peptide inhibitor of LIM-only protein 2 (Lmo2) targets zinc finger domains in a non-specific manner. Protein Pept Lett 21(2):132–139
29. Boerma EG, Siebert R, Kluin PM, Baudis M (2008) Translocations involving 8q24 in Burkitt lymphoma and other malignant lymphomas: a historical review of cytogenetics in the light of todays knowledge. Leukemia 23(2):225–234
30. Brady G, MacArthur GJ, Farrell PJ (2008) Epstein–Barr virus and Burkitt lymphoma. Postgrad Med J 84(993):372–377
31. Adhikary S, Eilers M (2005) Transcriptional regulation and transformation by Myc proteins. Nat Rev Mol Cell Biol 6(8):635–645
32. Grandori C, Cowley SM, James LP, Eisenman RN (2000) The Myc/Max/Mad network and the transcriptional control of cell behavior. Annu Rev Cell Dev Biol 16:653–699
33. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126(4):663–676
34. Dang CV, O'Donnell KA, Zeller KI, Nguyen T, Osthus RC, Li F (2006) The c-Myc target gene network. Semin Cancer Biol 16(4):253–264
35. Wang X, Zhao X, Gao P, Wu M (2013) c-Myc modulates microRNA processing via the transcriptional regulation of Drosha. Sci Rep 3:1942
36. Gregory MA, Hann SR (2000) c-Myc proteolysis by the ubiquitin-proteasome pathway: stabilization of c-Myc in Burkitt's lymphoma cells. Mol Cell Biol 20(7):2423–2435
37. Beverly LJ, Varmus HE (2009) MYC-induced myeloid leukemogenesis is accelerated by all six members of the antiapoptotic BCL family. Oncogene 28(9):1274–1279
38. Liu D, Shimonov J, Primanneni S, Lai Y, Ahmed T, Seiter K (2007) t(8;14;18): a 3-way chromosome translocation in two patients with Burkitt's lymphoma/leukemia. Mol Cancer 6:35
39. Patel JH, Loboda AP, Showe MK, Showe LC, McMahon SB (2004) Analysis of genomic targets reveals complex functions of MYC. Nat Rev Cancer 4(7):562–568. doi:10.1038/nrc1393
40. Meyer N, Penn LZ (2008) Reflecting on 25 years with MYC. Nat Rev Cancer 8(12):976–990. doi:10.1038/nrc2231
41. Klapproth K, Wirth T (2010) Advances in the understanding of MYC-induced lymphomagenesis. Br J Haematol 149(4):484–497
42. Hecht JL, Aster JC (2000) Molecular biology of Burkitt's lymphoma. J Clin Oncol 18(21):3707–3721
43. Wang H, Hammoudeh DI, Follis AV, Reese BE, Lazo JS, Metallo SJ et al (2007) Improved low molecular weight Myc-Max inhibitors. Mol Cancer Ther 6(9):2399–2408
44. Yin X, Giap C, Lazo JS, Prochownik EV (2003) Low molecular weight inhibitors of Myc-Max interaction and function. Oncogene 22(40):6151–6159

45. Follis AV, Hammoudeh DI, Wang H, Prochownik EV, Metallo SJ (2008) Structural rationale for the coupled binding and unfolding of the c-Myc oncoprotein by small molecules. Chem Biol 15(11):1149–1155
46. Clausen DM, Guo J, Parise RA, Beumer JH, Egorin MJ, Lazo JS et al (2010) In vitro cytotoxicity and in vivo efficacy, pharmacokinetics, and metabolism of 10074-G5, a novel small-molecule inhibitor of c-Myc/Max dimerization. J Pharmacol Exp Ther 335(3):715–727
47. Fletcher S, Prochownik EV (2015) Small-molecule inhibitors of the Myc oncoprotein. Biochim Biophysi Acta 1849(5):525–543
48. Goff SP, Gilboa E, Witte ON, Baltimore D (1980) Structure of the Abelson murine leukemia virus genome and the homologous cellular gene: studies with cloned viral DNA. Cell 22(3):777–785
49. Hantschel O, Superti-Furga G (2004) Regulation of the c-Abl and Bcr-Abl tyrosine kinases. Nat Rev Mol Cell Biol 5(1):33–44. doi:10.1038/nrm1280
50. Nagar B, Hantschel O, Young MA, Scheffzek K, Veach D, Bornmann W et al (2003) Structural basis for the autoinhibition of c-Abl tyrosine kinase. Cell 112(6):859–871
51. Honda H, Hirai H (2001) Model mice for BCR/ABL-positive leukemias. Blood Cells Mol Dis 27(1):265–278
52. Maru Y, Witte ON (1991) The BCR gene encodes a novel serine/threonine kinase activity within a single exon. Cell 67(3):459–468
53. Diekmann D, Brill S, Garrett MD, Totty N, Hsuan J, Monfries C et al (1991) Bcr encodes a GTPase-activating protein for p21rac. Nature 351(6325):400–402. doi:10.1038/351400a0
54. Chuang TH, Xu X, Kaartinen V, Heisterkamp N, Groffen J, Bokoch GM (1995) Abr and Bcr are multifunctional regulators of the Rho GTP-binding protein family. Proc Natl Acad Sci U S A 92(22):10282–10286
55. Zhao X, Ghaffari S, Lodish H, Malashkevich VN, Kim PS (2002) Structure of the Bcr-Abl oncoprotein oligomerization domain. Nat Struct Biol 9(2):117–120
56. Kantarjian HM, Talpaz M, Dhingra K, Estey E, Keating MJ, Ku S et al (1991) Significance of the P210 versus P190 molecular abnormalities in adults with Philadelphia chromosome-positive acute leukemia. Blood 78(9):2411–2418
57. Suryanarayan K, Hunger S, Kohler S, Carroll A, Crist W, Link M et al (1991) Consistent involvement of the bcr gene by 9;22 breakpoints in pediatric acute leukemias. Blood 77(2):324–330
58. Wilson G, Frost L, Goodeve A, Vandenberghe E, Peake I, Reilly J (1997) BCR-ABL transcript with an e19a2 (c3a2) junction in classical chronic myeloid leukemia. Blood 89(8):3064
59. McWhirter JR, Galasso DL, Wang JY (1993) A coiled-coil oligomerization domain of Bcr is essential for the transforming function of Bcr-Abl oncoproteins. Mol Cell Biol 13(12):7587–7595
60. Muller AJ, Young JC, Pendergast AM, Pondel M, Landau NR, Littman DR et al (1991) BCR first exon sequences specifically activate the BCR/ABL tyrosine kinase oncogene of Philadelphia chromosome-positive human leukemias. Mol Cell Biol 11(4):1785–1792
61. Pendergast AM, Muller AJ, Havlik MH, Maru Y, Witte ON (1991) BCR sequences essential for transformation by the BCR-ABL oncogene bind to the ABL SH2 regulatory domain in a non-phosphotyrosine-dependent manner. Cell 66(1):161–171
62. Greuber EK, Smith-Pearson P, Wang J, Pendergast AM (2013) Role of ABL family kinases in cancer: from leukaemia to solid tumours. Nat Rev Cancer [Review] 13(8):559–571
63. Ren R (2005) Mechanisms of BCR-ABL in the pathogenesis of chronic myelogenous leukaemia. Nat Rev Cancer 5(3):172–183. doi:10.1038/nrc1567
64. Bose S, Deininger M, Gora-Tybor J, Goldman JM, Melo JV (1998) The presence of typical and atypical BCR-ABL fusion genes in leukocytes of normal individuals: biologic significance and implications for the assessment of minimal residual disease. Blood 92(9):3362–3367
65. Druker BJ, Tamura S, Buchdunger E, Ohno S, Segal GM, Fanning S et al (1996) Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. Nat Med 2(5):561–566

66. Trela E, Glowacki S, Blasiak J (2014) Therapy of chronic myeloid leukemia: twilight of the imatinib era? ISRN Oncol 2014:596483
67. Bakhshi A, Jensen JP, Goldman P, Wright JJ, McBride OW, Epstein AL et al (1985) Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around JH on chromosome 14 and near a transcriptional unit on 18. Cell 41(3):899–906
68. Tsujimoto Y, Cossman J, Jaffe E, Croce CM (1985) Involvement of the bcl-2 gene in human follicular lymphoma. Science 228(4706):1440–1443
69. Cleary ML, Smith SD, Sklar J (1986) Cloning and structural analysis of cDNAs for bcl-2 and a hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation. Cell 47(1):19–28
70. Dyer MJ, Zani VJ, Lu WZ, O'Byrne A, Mould S, Chapman R et al (1994) BCL2 translocations in leukemias of mature B cells. Blood 83(12):3682–3688
71. Aventin A, Mecucci C, Guanyabens C, Brunet S, Soler J, Bordes R et al (1990) Variant t(2;18) translocation in a Burkitt conversion of follicular lymphoma. Br J Haematol 74(3):367–369
72. Leroux D, Monteil M, Sotto JJ, Jacob MC, Le Marc'Hadour F, Bonnefoi H et al (1990) Variant t(2;18) translocation in a follicular lymphoma. Br J Haematol 75(2):290–292
73. Adachi M, Tefferi A, Greipp PR, Kipps TJ, Tsujimoto Y (1990) Preferential linkage of bcl-2 to immunoglobulin light chain gene in chronic lymphocytic leukemia. J Exp Med 171(2):559–564
74. Youle RJ, Strasser A (2008) The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol 9(1):47–59. doi:10.1038/nrm2308
75. Akao Y, Otsuki Y, Kataoka S, Ito Y, Tsujimoto Y (1994) Multiple subcellular localization of bcl-2: detection in nuclear outer membrane, endoplasmic reticulum membrane, and mitochondrial membranes. Cancer Res 54(9):2468–2471
76. Vaux DL, Cory S, Adams JM (1988) Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature 335(6189):440–442
77. Taylor RC, Cullen SP, Martin SJ (2008) Apoptosis: controlled demolition at the cellular level. Nat Rev Mol Cell Biol 9(3):231–241. doi:10.1038/nrm2312
78. Czabotar PE, Westphal D, Dewson G, Ma S, Hockings C, Fairlie WD et al (2013) Bax crystal structures reveal how BH3 domains activate Bax and nucleate its oligomerization to induce apoptosis. Cell 152(3):519–531
79. Dlugosz PJ, Billen LP, Annis MG, Zhu W, Zhang Z, Lin J et al (2006) Bcl-2 changes conformation to inhibit Bax oligomerization. EMBO J 25(11):2287–2296
80. Shortt J, Johnstone RW (2012) Oncogenes in cell survival and cell death. Cold Spring Harb Perspect Biol 4(12)
81. Laulier C, Lopez BS (2012) The secret life of Bcl-2: apoptosis-independent inhibition of DNA repair by Bcl-2 family members. Mutat Res Rev Mutat Res 751(2):247–257
82. Bai L, Wang S (2014) Targeting apoptosis pathways for new cancer therapeutics. Annu Rev Med 65:139–155

Chapter 22
Aberrant Transcriptional Programming in Blood Cancers

Constanze Bonifer, Peter N. Cockerill, and Anetta Ptasinska

Contents

22.1 The Epigenetic Regulatory Machinery 468
22.1.1 The Interplay of Transcription Factors with the Chromatin Landscape Drives Cell Differentiation 468
22.1.2 The Epigenetically Silent State Is Self-Sustaining 469
22.1.3 Changes in Transcription Factor and Chromatin Assemblies Occur in Stages 469
22.1.4 Polycomb Complexes Fine-Tune Differential Gene Expression 470
22.2 Mechanisms of Cancer Transcriptional and Epigenetic Deregulation in Cancer 471
22.2.1 Aberrant Gene Silencing 472
22.2.2 Aberrant Gene Activation 473
22.3 System-Wide Studies of Cancer Development 475
22.3.1 The Impact of Leukaemogenic Transcription Factor Complexes on the Chromatin Landscape 476
22.4 Outlook 478
Acknowledgements 479
References 479

Abstract Cancer is by definition a disease caused by mutations in genes that control cell differentiation and growth, leading to disruption of the normal differentiation programme and the generation of an abnormal cell type. Cellular identity is intrinsically controlled by the interplay of transcription factors with the chromatin modification and remodelling machinery. In cancer, this whole process is dysregulated, and it is abundantly clear from the types of mutations present that cancer is a disease of epigenetic reprogramming and transcriptional malfunction. In this review, we will use blood cancers as a paradigm to describe the different mechanisms by which transcriptional programming of chromatin and, thus, cell differentiation are derailed in cancer cells.

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22.1 The Epigenetic Regulatory Machinery

22.1.1 The Interplay of Transcription Factors with the Chromatin Landscape Drives Cell Differentiation

Cellular identities are defined by specific differential gene expression patterns, involving genes encoding regulatory factors such as signalling molecules and sequence-specific transcription factors. Cell differentiation is initiated by specific signals coming from outside the cell that act via receptors and terminate at responsive transcription factors which then switch genes on or off. Each cell type displays a specific transcriptional network which is the union of all cis-regulatory elements controlling gene expression and the combinations of transcription factors binding to these elements which define the regulatory logic by which genes interact and are controlled by outside signals [1]. Epigenetic modifiers and chromatin remodelers play a central role in gene regulation because most of the genome is packaged into nucleosomes and exists together with various non-histone proteins in a highly condensed inaccessible form. Accordingly, one of the main functions of transcription factors in the process of gene activation is the recruitment of chromatin modifiers that are able to evict nucleosomes and render gene regulatory elements accessible to other elements of the transcription apparatus. To activate genes, transcription factors recruit co-factors, such as histone acetylases and chromatin remodelling complexes which modify and evict nucleosomes, thus paving the way for the assembly of a full transcription factor complex that is capable of interacting with the basal transcription machinery and drive mRNA synthesis [2, 3]. Conversely, repressor molecules recruit co-repressor complexes containing histone deacetylases which remove such modifications. This attracts chromatin remodelers, such as those of the of the BRG1/SMARCD3 (SWI/SNF) and SNF2H (ISWI) families which then use ATP to either slide nucleosomes away from transcription factor binding sites or, conversely, to evict transcription factor complexes [4]. However, this simple scenario does not reflect the true complexity of gene activation and gene silencing. Besides histone acetylation, histone tails are modified by a bewildering variety of modifications, such as methylation, ubiquitination and phosphorylation, to name the most important ones, all of which are installed by enzymatic activities that have specific, but often

overlapping activities. An important principle is that these DNA and histone modifications are recognised by specific interacting complexes that serve to reinforce either the activated or the silent transcriptional state. Further details of how chromatin structure interacts with transcription factors and transcriptional co-factors to regulate gene expression during cell differentiation can be found in many excellent reviews [3, 5, 6]. In the context of this review, we will only highlight a few important principles.

22.1.2 The Epigenetically Silent State Is Self-Sustaining

The first principle is that in the absence of transcription factors destined to activate them, genes adopt a silent state. Stably silenced, or heterochromatic genes display specific chromatin features and, in addition, their DNA is methylated at CG-dinucleotides. This involves a conversion to 5-methylcytosine on both DNA strands at CG sequences which is introduced by DNA-methyltransferases (DNMTs). DNMT1 is regarded as a maintenance methylase because it associates with the DNA-replication machinery, and it requires a methylated cytosine on one strand of newly replicated DNA. By this means, it maintains the previously installed methylation states during replication thus restoring the original methylated state after cell division [7]. A correct level of DNMT1 is required for normal cell differentiation as exemplified in the development of multiple defects in the haematopoietic system of mice haploinsufficient for *Dnmt1* [8]. In contrast, DNMT3A and DNMT3B can be recruited to sites not previously methylated to establish new patterns of cytosine methylation.

Methylated DNA binds methyl-binding proteins that interact with histone-deacetylases and is associated with high levels of histones methylated at lysine 9 (H3K9) [9], which, in turn creates a binding site for heterochromatin protein 1 (HP1) [10, 11]. Methyl-binding proteins and HP1 synergise by recruiting histone deacetylases and H3K9 methyltransferases, thus forming highly cooperative assemblies that maintain and propagate the silenced state during cell division [12–14]. The result of these interactions is that heterochromatin is both highly condensed and self-sustaining.

22.1.3 Changes in Transcription Factor and Chromatin Assemblies Occur in Stages

The second principle is that both gene activation and gene silencing are multi-step processes that involve the cooperation of multiple factors. Active genes are bound by hundreds of transcription factors, all of which bind to specific sequences and their associated co-factors. The specific sequence composition of cis-regulatory

elements dictates the affinity of transcription factor binding and thus the pattern of local histone modifications [15, 16]. The activation of genes from the fully heterochromatic state requires the recruitment of factor complexes that remove the methyl groups from DNA. For many years, such an activity was elusive, but we now know that different mechanisms can contribute to the demethylation of DNA. One of the mechanisms that is of relevance in this review involves the TET-proteins which convert 5-methyl-cytosine into 5-hydroxy-methyl cytosine, a modification which is recognised by the DNA repair machinery and which is then converted through several intermediates to the original cytosine [17]. Once DNA is demethylated, it can be bound by transcription factors which recruit co-activators that counteract the activity of repressive activities. In addition, CGs within the binding sites of transcription factors are being directly protected from methylation by the binding of these factors [18–20]. In the presence of the full complement of active transcription factors specific for a given cell type, genes stay active even through multiple rounds of cell division as some chromatin modifiers and transcription factors have the ability to be retained on mitotic chromatin and are thought to serve as a nucleating signal for the reassembly of the transcriptional machinery [21–23].

22.1.4 Polycomb Complexes Fine-Tune Differential Gene Expression

The third principle regards the intermediate state of genes which are in the process of being switched on and off during development. Cell fate changes and alterations in the composition of transcription factor and chromatin complexes do not function like binary switches with all factors simultaneously falling off when a gene is being silenced or simultaneously assembling when it is activated. Cell fate changes in development occur gradually and involve the step-wise opening up of chromatin by the sequential assembly of factors on multiple cis-regulatory elements in the absence or low level of transcription, a process that is called priming [24, 25]. Conversely, the closure of chromatin of a previously active gene also occurs gradually, together with the changing active transcription factor composition within the cell [26, 27]. However, actual mRNA synthesis can cease very quickly and genes can exist in a plastic state where they can either be activated or further repressed, depending on signals and developmental cues. The formation of this plastic state is mediated by polycomb group (PcG) complexes, which consist of a vast zoo of multiple different subunits, but essentially come in two flavours, PRC1 and PRC2 [28, 29]. PRC2 carries at its heart a histone modification activity (EZH2 or EED) that deposits one, two or three methyl groups on histone H3K27. Work in mouse ES cells has shown that about 70 % of all H3 molecules contain the H3K27me2 mark, whereas H3K27me1 marks transcribed genes, and H3K27me3 is found at the promoters of genes that are poised for transcription, but are not active [30]. The H3K27me3 mark creates a binding site for the chromodomain within the CBX family (Polycomb) subunit of

PRC1 which stably associates with PRC2 in chromatin. The RING1 family component of PRC1 then deposits a ubiquitin molecule at Histone H2A. The end result of these interactions is a block in the activity of RNA-Polymerase II and transcription is halted even in the presence of bound transcriptional activators and the basal transcription machinery on the gene [31]. Such poised promoters often carry an active chromatin mark, H3K4me3, whose deposition is catalysed by the Mixed Lymphocyte Leukaemia (MLL/KMT2A) complex [32]. The MLL complex is required for transcription in every cell and interacts with factors which are required for transcriptional elongation, such as PAF1 and the super-elongation (SEC) complex [33, 34], which is the precise activity PcG complexes are interfering with. This ability of PcG complexes to counteract the action of activators and to hold genes in "limbo" is essential for the establishment of specific patterns of gene expression because mutation of core PcG units leads to the aberrant activation of developmental regulator genes and deregulation of differentiation [31]. In summary, PcG complexes are essential for dynamic cell differentiation as they ensure that gene regulation is fine-tuned and genes are activated and silenced at the appropriate differentiation stage.

22.2 Mechanisms of Cancer Transcriptional and Epigenetic Deregulation in Cancer

There is now overwhelming evidence that genetic changes in cancer cells disturb the finely tuned balance of the interaction of the transcription regulatory machinery with the genome. For leukaemia this has been impressively highlighted by The Cancer Genome Atlas consortium which demonstrated that the majority of mutations causing acute myeloid leukaemia (AML) affect members of the epigenetic regulatory machinery and signalling component. Furthermore, individual leukaemias carrying different mutations display different gene expression profiles [35]. A similar picture affecting different genes is now also emerging in other blood cancers, such as lymphoma [36], T-cell precursor acute lymphoblastic leukaemia [37] and even in solid tumours, such as breast cancers [38]. Cells can respond to such mutagenic events in multiple ways, none of which are mutually exclusive [39, 40]. They may display uncontrolled growth by chronically activating growth factor signals, which subvert the transcriptional control of cell cycle regulator genes. If a mutation occurs in the stem cell compartment, differentiation can be blocked and cells reside for too long in the transiently amplifying precursor compartment. In both cases this can lead to further mutations and genetic instability. A third type of mechanism contributing to leukaemia involves the activation of lineage inappropriate gene expression programmes which help cancer cells to evade normal developmental control mechanisms within their normal cellular context. This section will highlight some of these specific mechanisms in more detail.

22.2.1 Aberrant Gene Silencing

As described in Section A, cell differentiation involves the selective activation and silencing of genes. The complement of expressed transcription factors together with co-activators and co-repressors determines which genes are on or off. It is therefore not surprising that mutations in any of the members of the epigenetic regulatory machinery will have a profound influence on this process. The result is that genes that should be active are not and genes that should be silenced stay active. This was first observed in studies that demonstrated aberrant DNA-methylation of tumour suppressor genes [41], but was then extended by genome-wide studies to show that DNA-methylation patterns in cancer cells are highly indicative for specific cancer types, as exemplified by the display of differential methylation patterns in AML and lymphomas [42–45]. However, the details of the precise mechanism of how aberrant DNA methylation is established are only just beginning to be unravelled and, in most cases, the molecular mechanism is still unclear [46]. One way to generate a genome wide hypermethylation signature is by interfering with the molecular machinery that removes methyl groups from CG di-nucleotides as exemplified in patients that carry mutations in the TET2 pathway [47, 48]. Another way of attracting *de novo* methylation is lack of binding of transcription factors as there is an inverse correlation between DNA-methylation and transcription factor binding [49] as well as with the resistance to de novo methylation in cancer cells and transcription factor association [50, 51]. An interesting observation was that the majority of aberrantly methylated CG island promoters were targets of polycomb complexes in embryonic stem cells, but also in other stem cell types [52–54], indicating that non-expressed genes in "transition" carrying a poised RNA-Polymerase – MLL/KMT2A complex as well as bivalent histone marks are particularly vulnerable to epigenetic silencing. It has indeed been shown that loss of MLL/KMT2A from a CG island promoter leads to rapid loss of RNA-Polymerase binding followed by de novo DNA-methylation [55, 56], indicating that DNA-methylation is secondary to the cessation of transcription.

Many of the mutations in blood cell malignancies do not affect global transcriptional regulators, but alter the functions of specific transcription factors required to drive the development of specific haematopoietic lineages. Without these factors, development is blocked and cells become vulnerable to transformation [57]. Examples of this notion are mutations of *RUNX1, GATA2, SPI1/PU.1* and *CEBPA*, all of which influence lineage-specific terminal differentiation and are recurrent features in leukaemia. Experiments with knock-out mice have shown that blood cell development is absent in the absence of RUNX1 [58], whereas the loss of RUNX1 past the haematopoietic stem cell (HSC) stage does not impact on stem cell numbers, but instead mice eventually succumb to a myelodysplastic syndrome (MDS) or lymphoma [59]. In addition, the expression of RUNX1 mutants found in patients disturbs myeloid differentiation [60]. Similarly, GATA2 is required for HSC generation and survival, and mutations of *GATA* family transcription factors have been found in MDS and AML [61]. *GATA2* overexpression is incompatible with the

survival of leukaemic cells in a mouse model of AML, indicating that the reduction of GATA2 activity is required for leukaemogenesis [62]. SPI1/PU.1 is absolutely required for myelopoiesis [63], and in mouse models a reduction in the levels of this transcription to 20 % or its original level was sufficient to trigger AML [64]. This is consistent with the finding of SPI1/PU.1 haploinsufficiency in human AML [65, 66]. *CEBPA* plays a central role in myeloid differentiation, and is required for the transition of the common myeloid progenitor to the granulocyte-macrophage progenitor stage. This factor is one the most frequently mutated transcription factors in AML, and is frequently dysregulated [67]. C/EBPα function can be interfered with by either mutation of the gene or by epigenetic silencing by DNA-methylation [68–71]. Different truncated versions of C/EBPα are found in AML whose expression in mice leads to stem cell expansion and a myeloid differentiation block [69] which goes along with a failure to repress stem-cell specific genes [70].

In summary, from these data a picture emerges that highlights the central role of transcription factors, co-activators and PcG complexes in ensuring the balance between proliferation and differentiation and in protecting promoters from falling prey to DNA-methylation and stable epigenetic silencing.

22.2.2 Aberrant Gene Activation

With only very few exceptions, one mutation does not generate a tumour cell [72]. Multiple mutations are required for tumour formation and the reason for this may be the fact that tumour cells evolve to evade the multi-faceted control mechanisms by which an organism keeps uncontrolled cell growth at bay. Aberrant gene activation in cancer is likely a result of such a selection process. One of the hallmarks of malignant cells is that many tightly regulated growth-controlling signalling processes are constitutively active. This is achieved by the mutation of molecules involved in transmitting such signals into the nucleus or autocrine/paracrine stimulation of growth factor receptors. In blood cells, this involves cytokine receptors such as KIT and FLT3 as well as kinases such as ABL1 or JAK2, leading to the chronic activation of transcription factors integrating the response to such signals. For example, the direct activation of RAS pathways can lead to activation of genes, such as *CSF2/GM-CSF* and hypersensitivity to GM-CSF in myeloid malignancies via activation of the signalling-responsive transcription factors NFKB or AP1 [73, 74]. The mutation of Janus Kinase (JAK) results in constitutive phosphorylation of the STAT transcription factors and the up-regulation of STAT target genes [75]. Interestingly, JAK can also directly signal to chromatin by phosphorylating histones directly and excluding the binding of CBX5/HP1, thus helping to aberrantly activate self-renewal genes in leukaemia [76]. Last, but not least, it has recently been shown that chronic signalling from mutated growth factor receptors can directly impact on polycomb mediated silencing and allow the up-regulation of lineage inappropriate genes in AML [77].

An example for the complete epigenetic reprogramming of a cellular phenotype is classical Hodgkin lymphoma (HL). The malignant Reed-Sternberg cells of HL (HRS cells) originate from germinal centre B cells. Interestingly, HRS cells only make up only a small fraction of the actual tumour, the rest are normal cells of multiple lineages which may protect these cells from immune surveillance. HRS cells have lost much of their B cell specific gene expression programme and express multiple inflammatory response genes, chemokines and cytokines to which the surrounding cells respond [78]. The consequence of the chronic activation of inflammatory signals is that transcription factors linking such signals to gene expression control are constitutively active, with the most important factor being NF-κB, a feature which is shared with many cancers [79]. In addition, they express multiple lineage inappropriate genes, including the genes encoding the receptor for colony-stimulating-factor 1 (CSF1R or FMS) which is the main growth factor receptor for the macrophage lineage, and is normally only expressed in monocytes and macrophages [80]. Moreover, these cells also express CSF1 itself and this autocrine/paracrine loop is required for HL cell survival [81].

An interesting by-product of the activation of chronic signalling is the genome-wide activation of repeat elements. While cancer cells demonstrate hypermethylation of tumour suppressor genes, it has long been known that cancer cells display global hypomethylation [82]. This effect can mostly be attributed to the hypomethylation of repeat elements such as satellite DNAs, *ALU* elements and *LINE* elements [83]. These elements are normally methylated and packaged into heterochromatin during embryogenesis. Epigenetic silencing at this stage is efficiently enforced by DNA-methylation and the action of co-repressors that maintain the presence of inactive histone marks. It is now clear that repeat element activation has detrimental consequences for normal cells as activated repeat elements are a substrate for homologous recombination which has been shown to lead to genomic instability and translocations [84]. It was only recently shown that repeat element activation can also influence gene expression and tumour pathology [81, 85, 86]. In Hodgkin lymphoma, the aberrant expression of the *CSF1R* gene does not originate from its normal promoter, but from an aberrantly activated long terminal repeat (LTR) promoter of the *THE1B* family of repeats located 6.5 kb upstream of the normal transcription start site [81]. The activation of *THE1B* elements in HL cells is not restricted to the *CSF1R* locus, but occurs at many genomic locations. The molecular mechanism of this activation is a loss of expression of the co-repressor RUNX1T1I/MTG8/ETO by aberrant DNA-methylation. The link to signalling was established by the finding that *THE1B* elements contain functional binding sites for inducible transcription factors, including NF-κB, which are required to activate LTR-driven promoter activity. The artificial recreation of this situation in non HL cells by the knock-down of MTG8 and the overexpression of a constitutively active NF-kB was sufficient to activate wide-spread LTR-driven expression, indicating that the chronic activation of inducible transcription factors and the loss of epigenetic control can override the safeguards that normally protect cells from the activation of LTR promoters. The result is a cell that originates from B cells, but has been selected to use growth factor signals that are normally specific for myeloid cells.

Another way of aberrantly activating inappropriate gene expression programmes is the mutation or epigenetic silencing of genes encoding repressors. A reduction in the level of DNMT3A expression in mice is a trigger for genome-wide hypomethylation and the development of lymphomas [87]. In humans, 22 % of all adult cases of AML have mutations in *DNMT3A* associated with loss or reduction of function [88] leading to an erosion of DNA-methylation [89], expansion of haematopoietic stem cells and an impediment of differentiation [90], which may underlie early changes in the development of AML [91]. Inactivating mutations of *EZH2* have been found in MDS [92], and mice with a conditional deletion of *EZH2* develop MDS with a global reduction of H3K27me3 and the de-repression of some PcG target genes. However, the precise link to leukaemogenesis is as yet unclear [93].

Some of the most frequent mutations leading to transcriptional deregulation in leukaemia occur in the H3K4 methyltransferase KMT2A/MLL1 translocations involving over 100 different partner genes have been identified. These are found in 5–10 % of AML and in approximately 70 % of infant acute lymphoblastic leukaemia (ALL). Interestingly, these fusion proteins have lost their histone methylase activity as well as other domains involved the control of MLL degradation and, instead, are directly fused to genes encoding components of the Super Elongation Complex (SEC), such as MLLT1/ENL or AFF1/AF4 [94–97]. The result of this loss is that the normal control mechanisms that regulate the activity of MLL/KMT2A are disrupted, and the recombined protein functions as a strong activator of transcriptional elongation. A consequence of this activity is the chronic up-regulation of expression of homeobox genes, such as *HOXA9*, which is incompatible with terminal haematopoietic differentiation [98]. Leukaemias with MLL/KMT2A translocations display a specific gene expression profile, which is characterized by multi-lineage gene expression, again indicating lineage inappropriate gene activation [99]. In summary, these examples highlight how the investigation of the transcriptional control of normal and malignant blood cell development has uncovered the important molecular players controlling haematopoiesis, leaving no doubt that blood cancers are fundamentally disorders of epigenetic and transcriptional reprogramming.

22.3 System-Wide Studies of Cancer Development

The previous paragraphs have concentrated on describing the role of individual factors in cancer pathology. We are still only at the very beginning of our understanding of how single mutations kick off a process that eventually culminates in a full-blown cancer. The main reason for this is the fact that we have only recently begun the process of collecting the system-wide data that allows us to compare cancer cells with normal cells and to make sense of the differences. However, these studies have already made it abundantly clear that in cancer not just a few genes are deregulated, but the entire transcriptional network of cancer cells has been reprogrammed to support the survival of malignant cells within an increasingly hostile

normal cellular environment. In this chapter, we will review examples of system-wide studies which shine a first light on this process.

22.3.1 The Impact of Leukaemogenic Transcription Factor Complexes on the Chromatin Landscape

As outlined above, a number of studies have collected global gene expression and DNA methylation data and used such data to classify different types of cancer, and to identify subtypes of individual diseases. Since many leukaemia fusion proteins directly affect histone modifiers, several studies have examined the chromatin landscape in cancer cells with the aim to unravel global alterations in chromatin programming. Several studies compared histone modification profiles between normal cells and different types of AML [62, 100, 101]. They obtained important information about the nature of the genomic targets that were altered in leukaemia cells and uncovered alterations in the regulation of important drivers of differentiation and self-renewal. However, very few studies have actually linked the chromatin landscape with the actual genomic targets of nuclear oncoproteins, let alone linked aberrant signalling processes with alterations of transcription factor assembly in the nucleus. Here we will highlight leukaemia with defects in the Core-Binding Factor (CBF) proteins as an example of how such studies pave the way for more a global understanding of how cancer cells maintain their aberrant phenotype. A large subclass of AML is associated with chromosomal rearrangements that result in disruption of the function of either the RUNX1 transcription factor [t(8;21)] or its binding partner CBFB [inv(16)] which together make up the CBF complex. CBFB interaction is required for high-affinity DNA binding of RUNX1 [102]. RUNX1 function is also disrupted by mutations within the *RUNX1* gene in 10 % of karyotypically normal AMLs [35].

In approximately 10 % of all AMLs, the DNA sequences encoding the DNA-binding domain of *RUNX1* are translocated from chromosome 21 to chromosome 8, and fused to the *RUNX1T1*/*ETO*/*MTG8* [103] gene, encoding a transcriptional repressor protein, resulting in the formation of the fusion protein RUNX1/RUNX1T1. This fusion protein lacks the transactivation domain of RUNX1, but still forms a heterodimer with CBFB. Like other oncogenes, RUNX1/RUN1T1 is not capable of causing leukaemia by itself, and the induction of fully developed AML in t(8;21) patients requires secondary genetic alterations [104]. As discussed in the first chapter, the expression of RUNX1/RUNX1T1 delays differentiation and, thus, makes the cells vulnerable to a second mutagenic event, which in most cases is a mutation in a growth-promoting signalling molecule, such as KIT [35]. Genome-wide analyses in t(8;21) cell lines and in primary leukaemia cells using ChIP sequencing (ChIP-seq) for the first time highlighted the genomic targets of RUNX1/RUNX1T1 and their chromatin features [105–108]. As it turned out, RUNX1/RUNX1T1 occupies thousands of binding sites and the influence of binding of this aberrant protein on gene expression and the chromatin landscape is extensive. In

t(8;21) leukaemia cell lines RUNX1/RUNX1T1 exists as a tetramer that associates with multiple haematopoietic regulators such as TAL1, ERG, FLI1 and LMO2, both in vitro and at its genomic targets. The interaction with other factors is required for leukaemogenicity of RUNX1/RUNX1T1 [106, 109]. Moreover, all t(8;21) AML cells retain an intact copy of *RUNX1*, which is required for cell survival, a feature common with other core-binding factor leukaemias [105, 110], and all RUNX1/RUNX1T1 binding sites are also bound by RUNX1 [105, 108]. Depletion of RUNX1/RUNX1T1 using a knock-down approach leads to a global redistribution of RUNX1 binding [108]. Furthermore, global gene expression profiling demonstrated that depletion of RUNX1/RUNX1T1 results in differentiation of t(8;21) cells, with genes encoding regulators of proliferation and cell cycle progression being down-regulated, and myeloid differentiation genes being up-regulated [108], indicating that the continuous presence of the oncogene is required to block differentiation. Down-regulated genes include crucial regulators of myelopoiesis, such as *CEBPA*, partly explaining the differentiation block in t(8;21) cells.

In vitro experiments have shown that the RUNX1/RUNX1T1 protein interacts with the NCOR and SIN3A co-repressors [111] turning the RUNX1 trans-activator into a genuine repressor. However, the binding of RUNX1/RUNX1T1 is associated with both up- and down-regulated genes and individual RUNX1/RUNX1T1 bound genomic sites recruit both histone acetylases (HATs) and HDACs [109, 112, 113] indicating a complex and dynamic network of transcription factor and co-factor interactions impacting on target gene regulation. Depletion of RUNX1/RUNX1T1 correlates with increased histone H3 lysine-9 (H3K9) acetylation [107, 108], which is consistent with a mostly repressive activity of the fusion protein. The consequences of the t(8;21) are, therefore, more wide-ranging than simple loss of function mutations within the *RUNX1* gene, as found in other subsets of AML.

In summary these experiments show that the binding of RUNX1/RUNX1T1 to its targets interferes with normal transcription factor assemblies and alters their function, thus leading to changes in histone modifications and a block in differentiation. Multiple pathways are altered, including those coding for signalling molecules and regulators of differentiation, such as C/EBPα. Moreover, the expression of RUNX1/RUNX1T1 causes t(8;21) cells to re-balance global gene expression by using the remaining intact RUNX1 protein to enhance self-renewal. Similar widespread transcriptional reprogramming has also been shown for another core-factor binding leukaemia, AML with the inv(16), which affects the gene encoding CBFB [114] or for APL caused by the expression of PML/RARA [115]. These finding show the extent to which the expression of a single aberrant transcription factor is capable of reprogramming the epigenetic landscape of a haematopoietic precursor cell and they demonstrate the monumental task ahead for therapy.

22.4 Outlook

Our most successful weapon against cancer cells is still chemotherapy which relies on the selective killing of fast-growing cells. This has now been complemented with epigenetic therapies that globally alter the DNA-methylation and histone modification status of cells in the hope that the balance between cell proliferation and cell differentiation can be restored. However, in spite of their success, these global approaches could be compared to an attempt to change a derailed ecosystem by globally poisoning it, with the consequence that what is left of healthy ecologic interactions is damaged as well. Moreover, cancer cells show clonal evolution and change phenotype [116], or originate from quiescent stem cells out of reach for chemotherapy [91]. Furthermore, even the same tumour type displays a huge diversity of mutations from which it is difficult to identify the actual "driver" mutations that has set the cell off on the path to become malignant. The t(8;21) and other such leukaemic mutations are prominent examples for such driver mutations, but in many cases of leukaemia and, in particular, in solid tumours, the picture is much less clear. From the data presented in the previous chapters, it is therefore obvious that we need to harness the power of system-wide studies to identify pathways upon which specific cancer cells depend and which can be targeted from multiple angles. Thus, a number of efforts are underway to integrate multiple types of high-throughput data and combine them with mathematic modelling approaches to reconstruct functional cellular networks and predict responses to manipulation [117]. These efforts will hugely increase our basic knowledge of tumour pathology and will identify molecules and pathways that are "druggable". With the inhibitor for the BCR-ABL1 kinase (Imatinib) which causes chronic myelogenous leukaemia (CML), we have a very successful drug against a specific signalling molecule [118]. The extraordinary success of Imatinib led to the develop of a whole family of related kinase inhibitors effective against not just BCR-ABL1, but other cancers with signalling defects as well, such as gastrointestinal stromal cancers that carry *KIT* mutations [119]. However the clinical success of other inhibitors of signalling molecules known to influence cancer pathology, such as the RAS pathway, has been limited. It therefore becomes increasingly clear that system-wide studies are required to find efficient combinations of different drugs for different types of targets, the most important ones being nuclear proteins. Efforts are now directed at directly targeting aberrant sequence-specific transcription factors by small molecules [120–122], aberrantly recruited chromatin modifiers [123–126], and also members of the basal transcription machinery that are hyperactive in cancer cells [127, 128]. A very exciting recent development is the development of methods by which chromatin modifications and DNA-methylation are erased in a locus-specific fashion [129, 130], allowing us to glimpse a possible future where aberrant silencing of drivers of differentiation and tumour suppressor genes may one day be reversed. Provided that researchers are still allowed to dig deep into the fundamental mechanisms of normal and malignant cell differentiation, exciting times are ahead.

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References

1. Davidson EH (2010) Emerging properties of animal gene regulatory networks. Nature 468(7326):911–920
2. de la Serna IL, Ohkawa Y, Imbalzano AN (2006) Chromatin remodelling in mammalian differentiation: lessons from ATP-dependent remodellers. Nat Rev Genet 7(6):461–473
3. Cockerill PN (2011) Structure and function of active chromatin and DNase I hypersensitive sites. FEBS J 278(13):2182–2210
4. Swygert SG, Peterson CL (2014) Chromatin dynamics: interplay between remodeling enzymes and histone modifications. Biochim Biophys Acta 1839(8):728–736
5. Voss TC, Hager GL (2014) Dynamic regulation of transcriptional states by chromatin and transcription factors. Nat Rev Genet 15(2):69–81
6. Chen T, Dent SY (2014) Chromatin modifiers and remodellers: regulators of cellular differentiation. Nat Rev Genet 15(2):93–106
7. Chen T, Li E (2006) Establishment and maintenance of DNA methylation patterns in mammals. Curr Top Microbiol Immunol 301:179–201
8. Broske AM, Vockentanz L, Kharazi S et al (2009) DNA methylation protects hematopoietic stem cell multipotency from myeloerythroid restriction. Nat Genet 41(11):1207–1215
9. Klose RJ, Bird AP (2006) Genomic DNA methylation: the mark and its mediators. Trends Biochem Sci 31(2):89–97
10. Lachner M, O'Carroll D, Rea S, Mechtler K, Jenuwein T (2001) Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature 410(6824):116–120
11. Bannister AJ, Zegerman P, Partridge JF et al (2001) Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. Nature 410(6824):120–124
12. Fritsch L, Robin P, Mathieu JR et al (2010) A subset of the histone H3 lysine 9 methyltransferases Suv39h1, G9a, GLP, and SETDB1 participate in a multimeric complex. Mol Cell 37(1):46–56
13. Smallwood A, Esteve PO, Pradhan S, Carey M (2007) Functional cooperation between HP1 and DNMT1 mediates gene silencing. Genes Dev 21(10):1169–1178
14. Schultz DC, Ayyanathan K, Negorev D, Maul GG, Rauscher FJ 3rd (2002) SETDB1: a novel KAP-1-associated histone H3, lysine 9-specific methyltransferase that contributes to HP1-mediated silencing of euchromatic genes by KRAB zinc-finger proteins. Genes Dev 16(8):919–932
15. McVicker G, van de Geijn B, Degner JF et al (2013) Identification of genetic variants that affect histone modifications in human cells. Science 342(6159):747–749
16. Kilpinen H, Waszak SM, Gschwind AR et al (2013) Coordinated effects of sequence variation on DNA binding, chromatin structure, and transcription. Science 342(6159):744–747
17. Solary E, Bernard OA, Tefferi A, Fuks F, Vainchenker W (2014) The Ten-Eleven Translocation-2 (TET2) gene in hematopoiesis and hematopoietic diseases. Leukemia 28(3):485–496
18. Kress C, Thomassin H, Grange T (2001) Local DNA demethylation in vertebrates: how could it be performed and targeted? FEBS Lett 494(3):135–140
19. Hsieh CL (1999) Evidence that protein binding specifies sites of DNA demethylation. Mol Cell Biol 19(1):46–56

20. Tagoh H, Melnik S, Lefevre P, Chong S, Riggs AD, Bonifer C (2004) Dynamic reorganization of chromatin structure and selective DNA demethylation prior to stable enhancer complex formation during differentiation of primary hematopoietic cells in vitro. Blood 103(8):2950–2955
21. Rada-Iglesias A (2013) Pioneering barren land: mitotic bookmarking by transcription factors. Dev Cell 24(4):342–344
22. Blobel GA, Kadauke S, Wang E et al (2009) A reconfigured pattern of MLL occupancy within mitotic chromatin promotes rapid transcriptional reactivation following mitotic exit. Mol Cell 36(6):970–983
23. Chen D, Dundr M, Wang C et al (2005) Condensed mitotic chromatin is accessible to transcription factors and chromatin structural proteins. J Cell Biol 168(1):41–54
24. Mansson R, Hultquist A, Luc S et al (2007) Molecular evidence for hierarchical transcriptional lineage priming in fetal and adult stem cells and multipotent progenitors. Immunity 26(4):407–419
25. Walter K, Bonifer C, Tagoh H (2008) Stem cell-specific epigenetic priming and B cell-specific transcriptional activation at the mouse Cd19 locus. Blood 112(5):1673–1682
26. Bonifer C, Hoogenkamp M, Krysinska H, Tagoh H (2008) How transcription factors program chromatin – lessons from studies of the regulation of myeloid-specific genes. Semin Immunol 20(4):257–263
27. Tagoh H, Schebesta A, Lefevre P et al (2004) Epigenetic silencing of the c-fms locus during B-lymphopoiesis occurs in discrete steps and is reversible. EMBO J 23(21):4275–4285
28. Simon JA, Kingston RE (2009) Mechanisms of polycomb gene silencing: knowns and unknowns. Nat Rev Mol Cell Biol 10(10):697–708
29. Morey L, Helin K (2010) Polycomb group protein-mediated repression of transcription. Trends Biochem Sci 35(6):323–332
30. Ferrari KJ, Scelfo A, Jammula S et al (2014) Polycomb-dependent H3K27me1 and H3K27me2 regulate active transcription and enhancer fidelity. Mol Cell 53(1):49–62
31. Di Croce L, Helin K (2013) Transcriptional regulation by Polycomb group proteins. Nat Struct Mol Biol 20(10):1147–1155
32. Milne TA, Briggs SD, Brock HW et al (2002) MLL targets SET domain methyltransferase activity to Hox gene promoters. Mol Cell 10(5):1107–1117
33. Milne TA, Kim J, Wang GG et al (2010) Multiple interactions recruit MLL1 and MLL1 fusion proteins to the HOXA9 locus in leukemogenesis. Mol Cell 38(6):853–863
34. Luo Z, Lin C, Shilatifard A (2012) The super elongation complex (SEC) family in transcriptional control. Nat Rev Mol Cell Biol 13(9):543–547
35. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia (2013) N Engl J Med 368(22):2059–2074
36. Okosun J, Bodor C, Wang J et al (2014) Integrated genomic analysis identifies recurrent mutations and evolution patterns driving the initiation and progression of follicular lymphoma. Nat Genet 46(2):176–181
37. Zhang J, Ding L, Holmfeldt L et al (2012) The genetic basis of early T-cell precursor acute lymphoblastic leukaemia. Nature 481(7380):157–163
38. Comprehensive molecular portraits of human breast tumours (2012) Nature 490(7418):61–70
39. Bonifer C, Bowen DT (2010) Epigenetic mechanisms regulating normal and malignant haematopoiesis: new therapeutic targets for clinical medicine. Expert Rev Mol Med 12:e6
40. Baylin SB, Jones PA (2011) A decade of exploring the cancer epigenome – biological and translational implications. Nat Rev Cancer 11(10):726–734
41. Greger V, Passarge E, Hopping W, Messmer E, Horsthemke B (1989) Epigenetic changes may contribute to the formation and spontaneous regression of retinoblastoma. Hum Genet 83(2):155–158
42. Figueroa ME, Lugthart S, Li Y et al (2010) DNA methylation signatures identify biologically distinct subtypes in acute myeloid leukemia. Cancer Cell 17(1):13–27

43. Saied MH, Marzec J, Khalid S et al (2012) Genome wide analysis of acute myeloid leukemia reveal leukemia specific methylome and subtype specific hypomethylation of repeats. PLoS ONE 7(3):e33213
44. Shaknovich R, Geng H, Johnson NA et al (2010) DNA methylation signatures define molecular subtypes of diffuse large B-cell lymphoma. Blood 116(20):e81–e89
45. Nordlund J, Backlin CL, Wahlberg P et al (2013) Genome-wide signatures of differential DNA methylation in pediatric acute lymphoblastic leukemia. Genome Biol 14(9):r105
46. Schoofs T, Berdel WE, Muller-Tidow C (2014) Origins of aberrant DNA methylation in acute myeloid leukemia. Leukemia 28(1):1–14
47. Figueroa ME, Abdel-Wahab O, Lu C et al (2010) Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. Cancer Cell 18(6):553–567
48. Ko M, Huang Y, Jankowska AM et al (2010) Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. Nature 468(7325):839–843
49. Thurman RE, Rynes E, Humbert R et al (2012) The accessible chromatin landscape of the human genome. Nature 489(7414):75–82
50. Gebhard C, Benner C, Ehrich M et al (2010) General transcription factor binding at CpG islands in normal cells correlates with resistance to de novo DNA methylation in cancer cells. Cancer Res 70(4):1398–1407
51. Schoofs T, Rohde C, Hebestreit K et al (2013) DNA methylation changes are a late event in acute promyelocytic leukemia and coincide with loss of transcription factor binding. Blood 121(1):178–187
52. Easwaran H, Johnstone SE, Van Neste L et al (2012) A DNA hypermethylation module for the stem/progenitor cell signature of cancer. Genome Res 22(5):837–849
53. Ohm JE, McGarvey KM, Yu X et al (2007) A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. Nat Genet 39(2):237–242
54. Schlesinger Y, Straussman R, Keshet I et al (2007) Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. Nat Genet 39(2):232–236
55. Ladopoulos V, Hofemeister H, Hoogenkamp M, Riggs AD, Stewart AF, Bonifer C (2013) The histone methyltransferase KMT2B is required for RNA polymerase II association and protection from DNA methylation at the MagohB CpG island promoter. Mol Cell Biol 33(7):1383–1393
56. Erfurth FE, Popovic R, Grembecka J et al (2008) MLL protects CpG clusters from methylation within the Hoxa9 gene, maintaining transcript expression. Proc Natl Acad Sci U S A 105(21):7517–7522
57. Rosenbauer F, Tenen DG (2007) Transcription factors in myeloid development: balancing differentiation with transformation. Nat Rev Immunol 7(2):105–117
58. Okuda T, van Deursen J, Hiebert SW, Grosveld G, Downing JR (1996) AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. Cell 84(2):321–330
59. Putz G, Rosner A, Nuesslein I, Schmitz N, Buchholz F (2006) AML1 deletion in adult mice causes splenomegaly and lymphomas. Oncogene 25(6):929–939
60. Cammenga J, Niebuhr B, Horn S et al (2007) RUNX1 DNA-binding mutants, associated with minimally differentiated acute myelogenous leukemia, disrupt myeloid differentiation. Cancer Res 67(2):537–545
61. Hahn CN, Chong CE, Carmichael CL et al (2011) Heritable GATA2 mutations associated with familial myelodysplastic syndrome and acute myeloid leukemia. Nat Genet 43(10):1012–1017
62. Bonadies N, Foster SD, Chan WI et al (2011) Genome-wide analysis of transcriptional reprogramming in mouse models of acute myeloid leukaemia. PLoS ONE 6(1):e16330

63. Koschmieder S, Rosenbauer F, Steidl U, Owens BM, Tenen DG (2005) Role of transcription factors C/EBPalpha and PU.1 in normal hematopoiesis and leukemia. Int J Hematol 81(5):368–377
64. Rosenbauer F, Wagner K, Kutok JL et al (2004) Acute myeloid leukemia induced by graded reduction of a lineage-specific transcription factor, PU.1. Nat Genet 36(6):624–630
65. Bonadies N, Pabst T, Mueller BU (2010) Heterozygous deletion of the PU.1 locus in human AML. Blood 115(2):331–334
66. Mueller BU, Pabst T, Osato M et al (2002) Heterozygous PU.1 mutations are associated with acute myeloid leukemia. Blood 100(3):998–1007
67. Paz-Priel I, Friedman A (2011) C/EBPalpha dysregulation in AML and ALL. Crit Rev Oncog 16(1–2):93–102
68. Fasan A, Haferlach C, Alpermann T et al (2013) The role of different genetic subtypes of CEBPA mutated AML. Leukemia 28(4):794–803
69. Bereshchenko O, Mancini E, Moore S et al (2009) Hematopoietic stem cell expansion precedes the generation of committed myeloid leukemia-initiating cells in C/EBPalpha mutant AML. Cancer Cell 16(5):390–400
70. Zhang H, Alberich-Jorda M, Amabile G et al (2013) Sox4 is a key oncogenic target in C/EBPalpha mutant acute myeloid leukemia. Cancer Cell 24(5):575–588
71. Hackanson B, Bennett KL, Brena RM et al (2008) Epigenetic modification of CCAAT/enhancer binding protein alpha expression in acute myeloid leukemia. Cancer Res 68(9):3142–3151
72. Vogelstein B, Kinzler KW (1993) The multistep nature of cancer. Trends Genet: TIG 9(4):138–141
73. Ward AF, Braun BS, Shannon KM (2012) Targeting oncogenic Ras signaling in hematologic malignancies. Blood 120(17):3397–3406
74. Parikh C, Subrahmanyam R, Ren R (2006) Oncogenic NRAS rapidly and efficiently induces CMML- and AML-like diseases in mice. Blood 108(7):2349–2357
75. Chen E, Staudt LM, Green AR (2012) Janus kinase deregulation in leukemia and lymphoma. Immunity 36(4):529–541
76. Dawson MA, Bannister AJ, Gottgens B et al (2009) JAK2 phosphorylates histone H3Y41 and excludes HP1alpha from chromatin. Nature 461(7265):819–822
77. Ray D, Kwon SY, Tagoh H, Heidenreich O, Ptasinska A, Bonifer C (2013) Lineage-inappropriate PAX5 expression in t(8;21) acute myeloid leukemia requires signaling-mediated abrogation of polycomb repression. Blood 122(5):759–769
78. Kuppers R (2009) The biology of Hodgkin's lymphoma. Nat Rev Cancer 9(1):15–27
79. Gasparini C, Celeghini C, Monasta L, Zauli G (2014) NF-kappaB pathways in hematological malignancies. Cell Mol Life Sci: CMLS 71(11):2083–2102
80. Bonifer C, Hume DA (2008) The transcriptional regulation of the colony-stimulating factor 1 receptor (csf1r) gene during hematopoiesis. Frontiers Biosci: J Virtual Libr 13:549–560
81. Lamprecht B, Walter K, Kreher S et al (2010) Derepression of an endogenous long terminal repeat activates the CSF1R proto-oncogene in human lymphoma. Nat Med 16(5):571–579, 571 p following 579
82. Feinberg AP, Tycko B (2004) The history of cancer epigenetics. Nat Rev Cancer 4(2):143–153
83. Ehrlich M (2009) DNA hypomethylation in cancer cells. Epigenomics 1(2):239–259
84. Robberecht C, Voet T, Zamani Esteki M, Nowakowska BA, Vermeesch JR (2013) Nonallelic homologous recombination between retrotransposable elements is a driver of de novo unbalanced translocations. Genome Res 23(3):411–418
85. Shukla R, Upton KR, Munoz-Lopez M et al (2013) Endogenous retrotransposition activates oncogenic pathways in hepatocellular carcinoma. Cell 153(1):101–111
86. Wolff EM, Byun HM, Han HF et al (2010) Hypomethylation of a LINE-1 promoter activates an alternate transcript of the MET oncogene in bladders with cancer. PLoS Genet 6(4):e1000917

87. Gaudet F, Hodgson JG, Eden A et al (2003) Induction of tumors in mice by genomic hypomethylation. Science 300(5618):489–492
88. Shih AH, Abdel-Wahab O, Patel JP, Levine RL (2012) The role of mutations in epigenetic regulators in myeloid malignancies. Nat Rev Cancer 12(9):599–612
89. Jeong M, Sun D, Luo M et al (2014) Large conserved domains of low DNA methylation maintained by Dnmt3a. Nat Genet 46(1):17–23
90. Challen GA, Sun D, Jeong M et al (2012) Dnmt3a is essential for hematopoietic stem cell differentiation. Nat Genet 44(1):23–31
91. Shlush LI, Zandi S, Mitchell A et al (2014) Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. Nature 506(7488):328–333
92. Ernst T, Chase AJ, Score J et al (2010) Inactivating mutations of the histone methyltransferase gene EZH2 in myeloid disorders. Nat Genet 42(8):722–726
93. Muto T, Sashida G, Oshima M et al (2013) Concurrent loss of Ezh2 and Tet2 cooperates in the pathogenesis of myelodysplastic disorders. J Exp Med 210(12):2627–2639
94. Neff T, Armstrong SA (2013) Recent progress toward epigenetic therapies: the example of mixed lineage leukemia. Blood 121(24):4847–4853
95. Slany RK (2009) The molecular biology of mixed lineage leukemia. Haematologica 94(7):984–993
96. Mueller D, Bach C, Zeisig D et al (2007) A role for the MLL fusion partner ENL in transcriptional elongation and chromatin modification. Blood 110(13):4445–4454
97. Mueller D, Garcia-Cuellar MP, Bach C, Buhl S, Maethner E, Slany RK (2009) Misguided transcriptional elongation causes mixed lineage leukemia. PLoS Biol 7(11):e1000249
98. Thorsteinsdottir U, Kroon E, Jerome L, Blasi F, Sauvageau G (2001) Defining roles for HOX and MEIS1 genes in induction of acute myeloid leukemia. Mol Cell Biol 21(1):224–234
99. Armstrong SA, Staunton JE, Silverman LB et al (2002) MLL translocations specify a distinct gene expression profile that distinguishes a unique leukemia. Nat Genet 30(1):41–47
100. Krivtsov AV, Feng Z, Lemieux ME et al (2008) H3K79 methylation profiles define murine and human MLL-AF4 leukemias. Cancer Cell 14(5):355–368
101. Wei Y, Chen R, Dimicoli S et al (2013) Global H3K4me3 genome mapping reveals alterations of innate immunity signaling and overexpression of JMJD3 in human myelodysplastic syndrome CD34+ cells. Leukemia 27(11):2177–2186
102. Wang Q, Stacy T, Miller JD et al (1996) The CBFbeta subunit is essential for CBFalpha2 (AML1) function in vivo. Cell 87(4):697–708
103. Miyoshi H, Kozu T, Shimizu K et al (1993) The t(8;21) translocation in acute myeloid leukemia results in production of an AML1-MTG8 fusion transcript. EMBO J 12(7):2715–2721
104. Yuan Y, Zhou L, Miyamoto T et al (2001) AML1-ETO expression is directly involved in the development of acute myeloid leukemia in the presence of additional mutations. Proc Natl Acad Sci U S A 98(18):10398–10403
105. Ben-Ami O, Friedman D, Leshkowitz D et al (2013) Addiction of t(8;21) and inv(16) acute myeloid leukemia to native RUNX1. Cell Rep 4(6):1131–1143
106. Martens JH, Mandoli A, Simmer F et al (2012) ERG and FLI1 binding sites demarcate targets for aberrant epigenetic regulation by AML1-ETO in acute myeloid leukemia. Blood 120(19):4038–4048
107. Saeed S, Logie C, Francoijs KJ et al (2012) Chromatin accessibility, p300, and histone acetylation define PML-RARalpha and AML1-ETO binding sites in acute myeloid leukemia. Blood 120(15):3058–3068
108. Ptasinska A, Assi SA, Mannari D et al (2012) Depletion of RUNX1/ETO in t(8;21) AML cells leads to genome-wide changes in chromatin structure and transcription factor binding. Leukemia 26(8):1829–1841
109. Sun XJ, Wang Z, Wang L et al (2013) A stable transcription factor complex nucleated by oligomeric AML1-ETO controls leukaemogenesis. Nature 500(7460):93–97

110. Goyama S, Schibler J, Cunningham L, Zhang Y, Rao Y, Nishimoto N, Nakagawa M, Olsson A, Wunderlich M, Link KA, Mizukawa B, Grimes HL, Kurokawa M, Liu PP, Huang G, Mulloy JC (2013) J Clin Invest 123(9):3876–3888. doi:10.1172/JCI68557
111. Amann JM, Nip J, Strom DK et al (2001) ETO, a target of t(8;21) in acute leukemia, makes distinct contacts with multiple histone deacetylases and binds mSin3A through its oligomerization domain. Mol Cell Biol 21(19):6470–6483
112. Follows GA, Tagoh H, Lefevre P, Hodge D, Morgan GJ, Bonifer C (2003) Epigenetic consequences of AML1-ETO action at the human c-FMS locus. EMBO J 22(11):2798–2809
113. Wang L, Gural A, Sun XJ et al (2011) The leukemogenicity of AML1-ETO is dependent on site-specific lysine acetylation. Science 333(6043):765–769
114. Mandoli A, Singh AA, Jansen PW et al (2013) CBFB-MYH11/RUNX1 together with a compendium of hematopoietic regulators, chromatin modifiers and basal transcription factors occupies self-renewal genes in inv(16) acute myeloid leukemia. Leukemia 28(4):770–778
115. Martens JH, Brinkman AB, Simmer F et al (2010) PML-RARalpha/RXR alters the epigenetic landscape in acute promyelocytic leukemia. Cancer Cell 17(2):173–185
116. Greaves M, Maley CC (2012) Clonal evolution in cancer. Nature 481(7381):306–313
117. Pe'er D, Hacohen N (2011) Principles and strategies for developing network models in cancer. Cell 144(6):864–873
118. Druker BJ (2008) Translation of the Philadelphia chromosome into therapy for CML. Blood 112(13):4808–4817
119. Essat M, Cooper K (2011) Imatinib as adjuvant therapy for gastrointestinal stromal tumors: a systematic review. Int J Cancer 128(9):2202–2214
120. Cerchietti LC, Ghetu AF, Zhu X et al (2010) A small-molecule inhibitor of BCL6 kills DLBCL cells in vitro and in vivo. Cancer Cell 17(4):400–411
121. Racanicchi S, Maccherani C, Liberatore C et al (2005) Targeting fusion protein/corepressor contact restores differentiation response in leukemia cells. EMBO J 24(6):1232–1242
122. Wichmann C, Chen L, Heinrich M et al (2007) Targeting the oligomerization domain of ETO interferes with RUNX1/ETO oncogenic activity in t(8;21)-positive leukemic cells. Cancer Res 67(5):2280–2289
123. Roe JS, Vakoc CR (2013) Oncogene interference through targeting of chromatin regulators. Cell Cycle 12(10):1475–1476
124. Yu W, Chory EJ, Wernimont AK et al (2012) Catalytic site remodelling of the DOT1L methyltransferase by selective inhibitors. Nat Commun 3:1288
125. Kim W, Bird GH, Neff T et al (2013) Targeted disruption of the EZH2-EED complex inhibits EZH2-dependent cancer. Nat Chem Biol 9(10):643–650
126. Deshpande AJ, Bradner J, Armstrong SA (2012) Chromatin modifications as therapeutic targets in MLL-rearranged leukemia. Trends Immunol 33(11):563–570
127. Dawson MA, Prinjha RK, Dittmann A et al (2011) Inhibition of BET recruitment to chromatin as an effective treatment for MLL-fusion leukaemia. Nature 478(7370):529–533
128. Zuber J, Shi J, Wang E et al (2011) RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia. Nature 478(7370):524–528
129. Maeder ML, Angstman JF, Richardson ME et al (2013) Targeted DNA demethylation and activation of endogenous genes using programmable TALE-TET1 fusion proteins. Nat Biotechnol 31(12):1137–1142
130. Mendenhall EM, Williamson KE, Reyon D et al (2013) Locus-specific editing of histone modifications at endogenous enhancers. Nat Biotechnol 31(12):1133–1136

Index

A

Activation-induced cytidine deaminase (AID), 33–35, 37, 40, 43, 55, 84, 141, 146, 160, 161
Acute biphenotypic leukemia/leukaemia (ABL), 225, 230, 231, 409
Acute leukaemia, 66, 109, 161, 201, 224–226, 229, 231, 236, 238, 432
Acute lymphoblastic leukemia/leukaemia (ALL), 5, 6, 8, 54–55, 109, 189–207, 284, 432–434, 456, 471, 475
Acute myeloid leukemia/leukaemia (AML), 4, 8, 58, 66, 194, 200, 201, 207, 215, 225, 226, 228–231, 235–237, 239, 241, 242, 253, 275–306, 369, 433, 436, 449, 456, 471–473, 475–477
Acute promyelocytic leukemia/leukaemia (APL), 5, 6, 66, 251–266, 288–290, 297, 477
Adenoma, 3, 358, 359, 361, 368
AICDA, 34–37, 55, 65, 160, 161
Alectinib, 409
ALL. *See* Acute lymphoblastic leukemia/leukaemia (ALL)
Alveolar rhabdomyosarcoma (ARMS), 5, 325–326
Amplification, 16–18, 21–23, 28, 38, 40–42, 55, 75, 80, 81, 85, 87–88, 96–97, 99, 100, 118, 147, 151, 161, 164, 170, 173, 176, 192, 193, 195, 211, 225, 229, 382, 387–389, 409
Anaplastic carcinomas (ATC), 359
Anaplastic lymphoma kinase (ALK), 253, 371, 403, 404, 406–411, 413
Aneuploidy, 16, 198
Apoptosis, 38, 39, 54, 63–65, 113, 114, 116, 122–124, 159, 160, 162, 163, 170, 200, 201, 205, 206, 253, 255–258, 262, 264, 276, 322, 325, 411, 455, 459–462
Arsenic, 256, 262–266
Arsenic trioxide (ATO), 122, 124, 254, 264, 289
Ataxia telangiectasia mutated (ATM), 28, 35, 39–43, 82, 86, 165, 363
Autonomously replicating sequence (ARS), 77

B

Bacterial artificial chromosomes (BACS), 19, 440
B cell lymphoma (BCL), 31, 35–39, 41, 42, 88, 157–176, 230, 432, 433, 437, 439, 453
BCL6, 35, 123, 160–163, 166, 169–175
BCR-ABL1, 28, 55, 58, 59, 61, 66, 108–118, 120–122, 124, 190, 202–204, 253, 280, 432, 434, 457–459, 478
Blast crisis, 109, 115–117, 203, 204, 237
Bone marrow (BM), 4, 55, 62, 63, 96, 109, 140, 173, 224, 231, 241, 255, 257, 258, 262, 276, 292, 431, 434, 436
Bosutinib, 121
BRD4, 149, 150, 224, 227, 233, 234, 241–242, 450
Breakage-fusion-bridge (BFB), 18, 28, 38, 42, 62, 80, 88
Bromodomain, 241, 242, 450
Burkitt lymphoma (BL), 4, 28, 158, 159, 166–168, 194, 253, 432, 453–456

J.D. Rowley et al. (eds.), *Chromosomal Translocations and Genome Rearrangements in Cancer*, DOI 10.1007/978-3-319-19983-2

C
Cancer, 3, 9, 54, 86, 87, 99, 100, 366, 368, 369, 382, 384, 386, 388, 390, 391, 394, 404, 418, 422, 424, 430, 476
Cas9, 440, 441
CBF. *See* Core-binding factor (CBF)
CCND1, 22, 23, 114, 142, 145, 146, 150, 152, 161, 162, 165, 359, 449
CCND3, 143, 152, 167
CD4+ T cells, 159, 160
Cell of origin, 158–160, 225, 228, 231–232, 327
Centroblastss (CBs), 159, 160, 166
Centrocytes (CCs), 159, 160
Ceritinib, 409
Chromatin alterations in cancer, 476
Chromosomal fragile sites, 31, 73–90
Chromosomal translocations, 27–43, 54, 66, 88, 110, 141, 157–176, 189–207, 225–229, 252, 253, 255, 266, 326, 334, 336, 361, 403–413, 429–441, 448, 449, 451, 453, 455, 456, 459, 462
Chromosome
  aberrations, 4–6, 96, 277, 279, 283, 284, 287, 288, 296, 297, 322
  rearrangements, 3–9, 18, 79, 287, 298, 323, 407, 413
  translocation, 53–66, 141, 276, 322, 458
Chromothripsis, 17, 18, 298
Chronic lymphocytic leukemic (CLL), 158, 164, 170, 174–175, 459
Chronic myelogenous leukemia/leukaemia (CML), 4–6, 8, 28, 66, 107–126, 253, 432, 434, 440, 456–459, 478
Chronic myeloid leukaemia, 107–126, 202, 392, 456
Class switch recombination (CSR), 30–35, 37, 38, 40–43, 140–142, 144, 159–161, 166, 176
Clinical outcome, 55, 101, 109, 276, 287, 290–306
CLL. *See* Chronic lymphocytic leukemic (CLL)
Clonal evolution, 53–66, 478
Clustered regularly interspaced short palindromic repeat (CRISPR), 440
CML. *See* Chronic myelogenous leukemia/leukaemia (CML)
C-NHEJ, 29, 32, 33, 35, 37–42
Colon, 80, 388
Common fragile sites, 73–90, 366
Comparative genomic hybridization (CGH), 19–20, 22, 23, 101, 147, 149, 150, 298, 387
Copy number, 16–23, 54–57, 75, 85, 86, 89, 95–101, 149, 161–164, 253, 418, 421, 423, 431
Core-binding factor (CBF), 238, 284, 288, 305, 476, 477
Cre, 230, 435–439
Crizotinib, 404, 407–410, 412
CSR. *See* Class switch recombination (CSR)
CTNNB1 (β-catenin), 115
Cyclin-CDK, 78
Cytogenetics, 4, 6, 16–20, 22–24, 42, 89, 98, 110, 118, 120, 142, 144, 147, 191, 192, 198, 206, 255, 276–286, 290–306, 322, 325, 336, 453, 459

D
Dark zone (DZ), 159, 160
Dasatinib, 120–122, 204, 206
Deletion, 4, 16–19, 22, 28–30, 32, 34, 35, 41, 43, 57, 58, 65, 74, 75, 77, 79, 80, 82, 85, 87, 89, 90, 98–99, 111, 116, 147, 148, 151, 160, 161, 164, 165, 168, 170, 171, 173, 174, 192–196, 198, 203–205, 211, 225, 229, 235–237, 240, 254, 258, 278, 283, 289, 297, 305, 325, 338, 384, 385, 387, 409, 420, 424, 425, 431, 436, 451, 475
Deregulation of gene expression in cancer, 471
Diffuse large B cell lymphoma (DLBCL), 35, 158, 159, 161, 163–165, 169–176, 439, 453, 459
DNA
  breaks, 55, 77, 81, 89, 161, 254, 366
  repair, 38, 79, 83, 84, 86, 90, 116, 117, 324, 392, 462, 470
  replication, 31, 77–86, 89, 116, 162, 163, 166, 469
DNA-methyltransferases (DNMTs), 260, 261, 459, 469
DOT1L, 202, 207, 224, 227, 228, 234, 239–240, 243
DZ. *See* Dark zone (DZ)

E
Early detection, 389, 394
EML4-ALK, 404–410

Epigenetics, 84, 86–87, 99, 164, 170, 172, 190, 207, 232, 253, 257, 258, 260–261, 264, 342, 468–469, 471–475, 477, 478
EPOR. *See* Erythropoietin receptor gene (EPOR)
Epstein Barr virus (EBV), 79, 83, 168, 453, 455
ERBB2, 17, 18, 100
Erythropoietin receptor gene (EPOR), 64, 195
ESRRA, 420–422
ETS genes, 7, 61, 199, 338, 341–346, 382–388, 391–394, 450, 451
ETV6-RUNX1, 54–66, 190, 198–199, 433, 434, 437, 439
Ewing sarcoma, 5, 6, 284, 322, 325, 333–348
EWS/ERG, 334, 342, 343, 346
EWS/ETV1, 342–343
EWS/ETV4, 342–343
EWS/FEV, 342–343
EWS/FLI, 334, 336–348, 450

F
FFPE, 19–21
FGFR3, 141–143, 146, 411, 412
FHIT. *See* Fragile Histidine Triad (FHIT)
Fluorescence in situ hybridization (FISH), 6, 18–19, 22–24, 59–61, 79–81, 88, 96, 100, 142, 144, 146, 147, 149–153, 206, 289, 293, 299, 306, 344, 346, 347, 366, 367, 384, 387, 408, 421, 439
FMS-related tyrosine kinase-3 (FLT3), 194, 207, 236, 259, 278, 286, 288, 299, 300, 302, 473
Follicular dendritic cells (FDC), 159
Follicular thyroid carcinomas (FTC), 359, 368
Founder lesion, 61, 66
FRA3B, 74, 75, 79–84, 86–89
FRA16D, 75, 79, 80, 82, 83, 86–88, 145
FRA2G, 75, 81
FRA7G, 75, 80–82, 87, 88
Fragile Histidine Triad (FHIT), 79, 83, 87, 88, 99
FRA1H, 81
FRA7H, 75, 80, 82
FUS/ERG, 336, 338, 343–344, 346
FUS/FEV, 336, 343–344, 346
Fusion genes, 5–7, 9, 16, 58, 60–66, 108, 109, 198–203, 229, 238, 277, 288, 289, 291, 321, 322, 324–326, 369, 371, 372, 384, 386, 404–407, 410, 412, 419, 420, 431–434, 436, 439, 440
Fusion oncogenes, 321–327, 368, 404
Fusion proteins, 28, 63, 109, 111, 112, 161, 162, 191, 198–200, 203, 206, 233, 237, 240, 243, 253, 255–259, 266, 291, 323, 324, 326, 327, 334, 336, 337, 339–345, 348, 386, 387, 393, 406, 410–413, 422, 432, 433, 435–439, 448–450, 458, 475–477

G
GATA, 197, 451, 472
Gene fusions, 3–9, 66, 161, 252, 253, 266, 276, 283, 288, 289, 296, 298, 299, 306, 322, 324, 346, 381–394, 404, 410–412, 422, 430, 431, 434, 435, 448
Genetic instability, 75, 77, 83, 85, 90, 471
Genetic lesions, 66, 158, 160–165, 167, 170–176, 193, 204, 205, 306, 358, 360, 366
Genomic instability, 18, 28, 37, 39–41, 84, 86–88, 90, 112, 115–117, 167, 298, 322, 474
Germinal center, 141

H
Haematopoietic stem cell, 63, 108, 202, 255, 434, 451, 472, 475
Hematopoietic/haematopoietic malignancies, 472
HER2/neu, 100
Histone deacetylases (HDACs), 84, 256, 341, 468, 469
Hodgkin lymphoma (HL), 8, 158, 164, 176, 474
Homogeneously staining region (HSR), 16, 96, 206, 229
Homologous recombination (HR), 29, 82, 115, 144, 431, 435, 436, 439, 440, 474
HOOK2, 421
HOX11 (TLX1and TLX3) Genes in T-ALL, 198
Hyperdiploid, 55, 59, 146, 194, 207, 292, 297
Hypodiploid, 146, 194, 297

I
Ibrutinib, 174
Imatinib, 55, 117–120, 204, 206, 392, 409, 459, 478
Immunoglobulin heavy chain (IGH), 5, 26, 28, 30–35, 37, 38, 40–43, 64, 66, 88, 140–152, 161, 165–167, 169, 171, 191, 194, 195, 200, 253, 382, 449, 453, 459

Immunoglobulin (IG) remodeling, 161, 166
In Utero, 55–60, 201, 226, 232

K
Karyotype, 5, 7, 18, 96, 142, 276, 277, 283, 285–287, 289, 290, 292–299, 302, 303, 305, 308
KIF5B-ALK, 405, 407
KIF5B-RET, 371, 405, 410–411
Kinases, 37, 39, 55, 65, 78, 82, 112, 117, 119–122, 124, 203, 204, 206, 237, 324, 361, 363, 364, 368, 371, 372, 386, 392, 406, 408–411, 413, 448, 450, 456, 457–459, 473, 478
KIT, 118, 120, 121, 286–288, 409, 473, 476, 478
KMT2A/MLL leukemia stem cells, 223–244
KMT2A-MLLT1, 192, 438
Knock-out, 472

L
LEDGF, 233, 234, 242
Leukaemia/leukemia, 28, 54, 58, 60, 61, 63, 64, 66, 85, 108, 111, 112, 122, 123, 126, 191, 199, 201–203, 207, 225, 226, 228, 230, 231, 232, 236–239, 241, 253, 254, 256–259, 261, 266, 276, 278, 286, 293, 295, 299, 322, 323, 336, 338, 369, 413, 432–434, 437, 439, 440, 453, 458, 459, 471–473, 475–478
Leukaemic/leukemic stem cell (LSC), 115, 207, 243
Leukemia diagnosis, 293
Light zone (LZ), 159
LIM, 196, 451
LMO1, 6, 35, 192, 196–198
LMO2, 6, 35, 191–193, 196–198, 201, 432, 433, 447, 450–453, 477
LMO1/LMO2 driven T-ALL, 196–198
Long terminal repeat (LTR), 196, 432, 474
Loss of heterozygosity (LOH), 16, 19–21, 80, 98, 99
LSC. *See* Leukaemic/leukemic stem cell (LSC)
Lymphocyte development, 31
Lymphoma, 8, 28, 31, 35, 37–39, 41, 42, 46, 85, 87, 88, 152, 157–176, 200, 230, 266, 284, 322, 323, 382, 406, 431–433, 437, 439, 449, 453, 459, 471, 472, 475
Lysine-specific demethylase 1 (LSD1), 207, 241, 341

M
Mantle cell lymphoma (MCL), 152, 158–162, 164–166, 449, 453
MDS. *See* Myelodysplastic syndrome (MDS)
Mechanism of instability, 84, 87, 90
MENIN, 244
Meningioma, 4
MGUS. *See* Monoclonal Gammopathy of Undetermined Significance (MGUS)
MicroRNAs (miRNAs), 63, 196, 236, 455
MiRNAs. *See* MicroRNAs (miRNAs)
MLL-PTD, 228, 229
MLPA. *See* Multiplex ligation-dependent probe amplification (MLPA)
MM. *See* Multiple myeloma (MM)
Molecular targeted therapy, 117, 348, 391–393, 404, 410, 411, 459
Monoclonal Gammopathy of Undetermined Significance (MGUS), 66, 140, 142, 143, 146, 147, 149, 151–153
Multiple myeloma (MM), 28, 66, 88, 139–153
Multiplex ligation-dependent probe amplification (MLPA), 20
Mutation, 3, 4, 7, 16, 19, 33, 37, 39, 41, 54, 60–61, 74, 82, 86, 87, 98, 99, 115–121, 140, 141, 145, 146, 159, 161–164, 166–168, 173, 175, 190, 193, 194, 198, 199, 204–207, 224–226, 228, 235, 242, 253, 258–261, 263, 276, 278, 286, 288, 290, 291, 298–302, 340, 342, 359, 368, 386, 388, 391, 404, 407–409, 419, 422, 430, 434, 437, 453, 462, 471–473, 478
Mutations in the epigenetic regulatory machinery, 471, 472
MYC, 5, 6, 37–43, 84, 85, 88, 97, 100, 113, 114, 139–153, 161–163, 165–168, 170, 174, 194–197, 203, 242, 253, 259, 264, 382, 432, 439, 449, 453–456, 462
MYCN, 17, 97, 100, 147, 148, 197
MYC oncogene, 5, 28, 35, 173, 192, 194, 253
Myelodysplastic syndrome (MDS), 8, 66, 284, 291, 292, 472, 475

N
Nilotinib, 120, 121
NOTCH1, 61, 165, 174, 192, 194, 197, 198
NOTCH2, 165

O
Oncogenes, 5, 17, 28, 29, 35, 38, 60, 80, 85–88, 96–99, 114, 118, 123, 124,

141–143, 146, 161, 165, 170, 172, 190–194, 253, 258, 321–327, 360–362, 368, 369, 382, 387, 404, 418, 430, 453, 456, 476, 477
Oncogenesis, 35, 334, 336, 340–344, 346, 348, 418, 462
Origin recognition complex (ORC), 77, 79
Ovarian, 80, 86, 388, 417–425

P
PAFc, 233–235, 243
Papillary thyroid carcinomas (PTC), 359, 361–363, 366–369, 371, 449
Partial tandem duplication (PTD), 225, 228, 278, 302
PAX3, 325, 326
Pc-G. *See* Polycomb group (Pc-G)
PCL. *See* Plasma cell leukemia (PCL)
PDC. *See* Poorly differentiated carcinomas (PDC)
Philadelphia (Ph) chromosome, 4, 5, 28, 108–111, 126, 190, 202–204, 432, 434, 449, 456–459
Plasma cell leukemia (PCL), 140, 142
Plasma cell neoplasm, 8
PLZF, 258
PML-RARA, 66, 225, 253–266, 282, 285, 288–290, 298
Polycomb group (Pc-G), 170, 232, 237–238, 327, 470
Ponatinib, 121
Poorly differentiated carcinomas (PDC), 359, 363
Preclinical models, 430
PRMT1, 233, 234, 241
Prognostication, 306
Prostate, 7–9, 28, 56, 65, 66, 80, 363, 381–394, 404, 413, 425, 430
Protein over-expression, 389, 449, 450
PTC. *See* Papillary thyroid carcinomas (PTC)
PTD. *See* Partial tandem duplication (PTD)

R
RA. *See* Retinoic acid (RA)
Radiations, 336, 359, 365–369, 392, 460
RAG, 32–38, 40, 41, 55, 65, 160–161, 192, 193, 204, 438
RAG-2, 35, 41, 88, 439
RAS/RAF family, 113, 383, 393
Reactive oxygen species (ROS), 31, 115, 117, 264, 265, 366
Rebastinib, 121
Recombinase, 124, 240, 435, 436, 438
Recombination signal sequence (RSS), 32–35, 37, 40, 192, 193
Reed-Sternberg cells (HRS), 474
RET, 357–372, 410–411, 449
Retinoic acid (RA), 254–265, 288, 449
Retrovirus, 97, 432
ROS. *See* Reactive oxygen species (ROS)
ROS1 fusion, 410, 413
Rowley, J.D., 85, 108, 202, 254, 276
RSS. *See* Recombination signal sequence (RSS)
RUNX1, 55, 56, 59, 61–63, 115, 196–199, 238, 253, 278, 282–284, 288, 299, 301, 305, 433, 437, 438, 451, 472, 476, 477

S
S-adenosylmethionine (SAM), 240
Sarcoma, 5, 6, 66, 284, 321–327, 333–348, 382, 437, 450
SCEs. *See* Sister chromatid exchanges (SCEs)
SCTN3, 421
SEC. *See* Super elongation complex (SEC)
SHM. *See* Somatic hypermutation (SHM)
Signal transduction, 192, 276, 291, 322, 363–365
SIN3a, 62, 196, 256, 477
Sister chromatid exchanges (SCEs), 75, 79, 82
Somatic hypermutation (SHM), 34, 35, 37, 140–142, 144, 145, 159–161, 164–166, 169, 173
SOX2, 97
STAT1, 114
STAT5, 114, 196, 203–205
SUMO, 262
Sumoylation, 261, 264, 265
Super elongation complex (SEC), 227, 228, 234, 471, 475
Synovial sarcoma, 284, 326–327, 437, 450

T
T(2;8), 5, 162, 166, 194, 453
T(8;14), 4, 150, 162, 163, 166, 167, 173, 192, 194, 253, 279, 449, 453
T(8;21), 4, 6, 225, 276, 277, 282, 284–288, 294–296, 299, 303, 305, 433, 438, 449, 476–478

T(8;22), 5, 162, 166, 194, 279, 453
T(9;22)(q34.1;q11.2), 55, 109, 203, 456
T(12;21), 54–62, 64, 65, 198, 199, 433
T(14;16), 88, 143, 144, 152
TAL1, 6, 35, 191–193, 195–197, 477
TALENs. *See* Transcription activator-like effector nucleases (TALENs)
TAL1-positive T-ALL, 191, 195–196
Targeted therapy, 100, 108, 117–121, 174, 207, 225, 239–244, 251–266, 289, 297, 348, 391–393, 404, 410, 411, 459
T-cell receptor (TCR), 6, 28, 32, 35, 88, 191–193, 195, 196, 253, 322, 323, 438, 448, 450, 451
TCF3/E2A-PBX1, 200, 225
TCF7L2, 422–424
TCGA. *See* The Cancer Genome Atlas (TCGA)
TCR. *See* T-cell receptor (TCR)
TES (TESTIN), 80
Tetracycline, 432, 433
TF. *See* Transcription factor (TF)
TGF-β, 199
The Cancer Genome Atlas (TCGA), 8, 418, 419, 421–423, 425, 471
Thyroid carcinoma, 6, 357–372
TKI. *See* Tyrosine kinase inhibitor (TKI)
TLX1, 191–193, 198, 206
TLX3, 192, 198, 206
TMPRSS2, 7, 382–384
TMPRSS2-ERG/TMPRSS2:ERG, 66, 384, 385, 388–390, 404, 425
Topoisomerase II, 31, 201, 226, 254
TP53, 28, 38, 39, 41, 42, 85, 98, 123, 124, 164, 165, 168, 170, 172, 174, 194, 200, 204, 257, 262, 290, 298, 326, 359, 392
Transcription activator-like effector nucleases (TALENs), 30, 232, 440, 441
Transcriptional regulation, 61, 165, 232–234, 324, 449, 453–456
Transcription factor (TF), 39, 61–63, 65, 114, 115, 123, 124, 149, 162, 166, 167, 176, 190, 191, 195, 198–202, 207, 238, 253, 284, 291, 322–324, 326, 338, 340–342, 344, 345, 348, 365, 382, 383, 387, 392, 393, 413, 424, 448–451, 454, 456, 468–470, 472–474, 476–478
Trithorax (trx), 201, 225, 232, 233, 237
Trithorax-group (Trx-G), 232
Trx. *See* Trithorax (trx)
TSG. *See* Tumour suppressor gene (TSG)
Tumor type specificity, 323
Tumour suppressor gene (TSG), 16, 17, 63, 80, 85, 87–89, 96, 98–99, 145, 164, 242, 278, 286, 360, 363, 472, 474, 478
Twins, 56, 58–61, 64, 201, 226
Tyrosine kinase, 108, 112, 117, 123, 124, 176, 194, 202–206, 253, 286, 360–365, 368, 369, 410–412, 432, 449, 450, 456, 458, 459
Tyrosine kinase inhibitor (TKI), 108, 109, 111, 117–122, 126, 204, 206, 207, 372

V
V(D)J, 28, 31–35, 37–43, 141, 161, 192, 193
VHL, 61, 99

W
WDR5, 233, 243
Whole genome sequencing, 19, 21–22, 144, 150, 371, 418, 422

X
XRCC4, 37, 38

Z
Zinc-finger nucleases (ZFNs), 30, 232, 440, 441