2 The Biology and Pathogenesis of Chronic Myeloid Leukaemia

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2.1 The Molecular Biology of CML

2.1.1 The t(9;22) Translocation and the BCR-ABL1 Gene

The Philadelphia (Ph) chromosome is formed by a reciprocal t(9;22)(q34;q11) translocation between the long arms of chromosomes 9 and 22, causing the juxtaposition of the BCR (breakpoint cluster region) and ABL1 (Abelson) genes. The *BCR-ABL1* fusion gene consists of the 5′ end of the BCR gene and the 3′ end of the ABL1 gene (Fig. 2.1a). The location of the BCR and ABL1 genomic breakpoints is highly variable, but the recombination usually involves fusion of intron 13 or 14 of BCR with a 140-kilobase (kb) region of ABL1 surrounding exons 1b and 2 (Fig. 2.1a) [1, 2]. Regardless of the breakpoint location on the ABL1 gene, mRNA splicing gives rise to major *BCR-ABL1* transcripts with e13a2 (BCR exon 13 and ABL1 exon 2) or e14a2 junctions, originally referred to as b2a2 and b3a2, respectively. Both transcripts result in the expression of a 210 kDa *BCR-ABL1* protein with a 75-amino acid difference. In <2% of chronic phase (CP)-CML, 'atypical' transcripts can form when the breakpoint occurs between exons 1 and 2 (e1a2 transcript) or exons 19 and 20 (e19a2) of BCR. Alternative atypical transcripts have also been described although even less frequently [3, 4].

There has been much debate regarding the consequence of a patient expressing either the e13a2 or e14a2 transcripts [2]. Before the tyrosine kinase inhibitor (TKI) era, most reports on large series refuted the importance of the BCR breakpoint [5–8]. However, a recent revival of this debate has found consistent evidence that patients with either the e14a2 transcript or both the e14a2 and e13a2 transcripts exhibit a higher platelet count, approximating 1.5 times higher than that in the e13a2 group [3, 9, 10]. Several laboratories have also found that patients with e14a2 transcripts achieve optimal ELN-defined responses more rapidly, including the deep

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R. Hehlmann (ed.), *Chronic Myeloid Leukemia*, Hematologic Malignancies,
https://doi.org/10.1007/978-3-030-71913-5_2

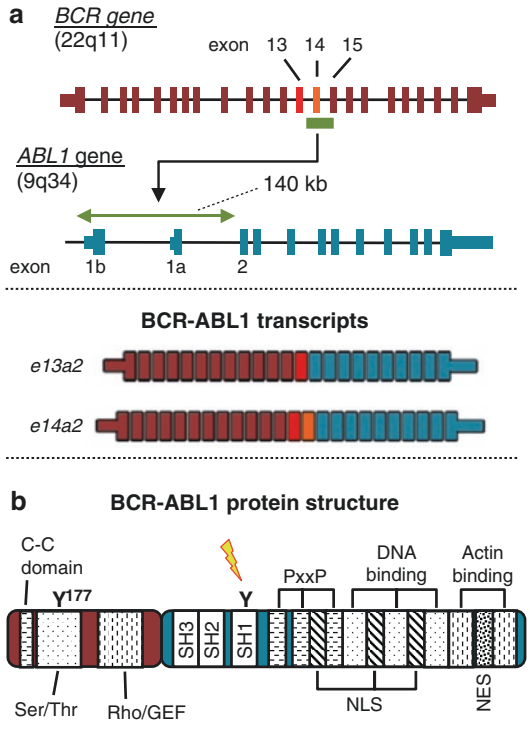

Fig. 2.1 The gene and protein structure of *BCR-ABL1*. (**a**) The *BCR-ABL1* fusion gene consists of the 5′ end of the *BCR* gene and the 3'end of *ABL1*. The location of the translocation usually involves fusion of intron 13 or 14 of *BCR* with a 140-kilobase (kb) region of *ABL1* surrounding exons 1b and 2. Examples of the two *BCR-ABL1* major mRNA isoforms are shown to highlight the *BCR* breakpoint variants. Depending on the breakpoint on the *ABL1* gene, exons 1a and/or 1b may be included in the primary transcript but are always excluded from the mRNA because they lack a splice acceptor sequence. (**b**) The BCR-ABL1 protein contains the dimerisation or coiled-coil (C-C), the Ser-Thr kinase and the Rho/GEF domains of BCR, as well as the SH-domains, Proline-rich (PxxP) nuclear localisation signal (NLS), DNA-binding nuclear export signal (NES) and Actin-binding domains from ABL. The ATP-binding site in the SH1 domain is indicated, highlighting the site of traditional tyrosine kinase inhibitor binding. The tyrosine residues in the Ser/Thr and SH1 kinase domains have been highlighted with a Y. The diagrams in A and B are not to scale

molecular response which is mandated for consideration of a treatment-free remission attempt [3, 11–13]. Patients expressing both transcripts tend to track with the e14a2 group [3, 10]. More recently, the transcript type has been demonstrated to influence long-term treatment-free remission outcomes with e14a2 expression correlating with higher treatment-free remission success [13, 14]. Furthermore, despite the rarity, atypical *BCR-ABL1* transcripts are generally associated with inferior outcomes [15, 16].

BCR-ABL1 has also been detected in healthy individuals with neither clinical nor laboratory evidence of CML [17–21]. With limited follow-up, these patients do not develop CML, likely due to these events being detected in terminally differentiated leukocytes as opposed to the leukaemic stem cell [19]. The absence of *BCR-ABL1* in the pluripotent stem cell explains the lack of proliferative potential, corroborating that this genetic event must develop in the leukaemic stem cell for CML to develop.

2.1.2 Protein Structure

The 210 kDa *BCR-ABL1* protein observed in CML contains more than ten protein domains (Fig. 2.1b). The SH1 tyrosine kinase region is the most studied due to its inherent role in CML pathogenesis and, consequently, the target for TKIs [22]. However other important regions include the SH2, SH3 and the N-terminal cap [23]. Myristoyl modification of the N-terminal cap permits the regulation of the kinase domain by SH2 and SH3 [24]. The fusion of BCR to ABL1 eventuates in loss of the N-terminal cap which results in constitutive activation of the SH1 kinase domain, inducing uncontrolled signal transduction and abnormal cellular proliferation [22]. TKIs, such as imatinib, compete with ATP for binding at the catalytic domain, inhibiting the phosphorylation of the tyrosine residues on substrates and impeding the downstream signalling effects of the oncogenic protein [22, 24]. In contrast, asciminib mimics the actions of the myristoyl site of the N-terminal cap, leading to the allosteric inhibition of *BCR-ABL1* [25].

2.1.3 The Consequence of BCR-ABL1

The *BCR-ABL1* protein gives rise to aberrant activation of cell signalling pathways and a shift to a micro-environment that is optimal for the development of leukaemia. For example, CML cells exhibit changes in growth-factor dependence, apoptosis, proliferation and cell adhesion [24]. These changes result in excessive proliferation of granulocytes, leading to the clinical features observed in CP-CML [26, 27]. The importance of *BCR-ABL1* signalling (particularly via the tyrosine kinase domain) is ultimately corroborated by the efficacy of TKI therapy.

BCR-ABL1 is a multi-faceted fusion gene with a marked effect on downstream signalling pathways, all of which promote the leukaemic phenotype observed in CML. Early work involving the transplantation of murine bone marrow transfected with *BCR-ABL1* induced a CML-like disease in transplanted mice [26, 28, 29]. Additional experiments confirmed the oncogenic potential of *BCR-ABL1* through gradual disruption of cellular differentiation, dysregulated proliferation, growth factor independence and interference of apoptosis through downstream signalling pathways [30–32]. Moreover, studies targeting *BCR-ABL1* by antisense oligonucleotides [33–36] demonstrated that *BCR-ABL1* was crucial for maintenance of the leukaemic process. These early observations underpinned the function of *BCR-ABL1* and provided affirmation that this fusion oncogene is the sole driver of CP-CML. However evolution to the more aggressive stages of CML is likely dependent on the cooperation of *BCR-ABL1* with other genetic events implicated in malignancy [37].

2.2 Important Pathways Affected by *BCR-ABL1* Activity

2.2.1 JAK/STAT

The JAK/STAT signalling pathway has been heavily implicated in leukaemogenesis, including the pathogenesis of CML [38]. *BCR-ABL1* augments activation of JAK2 through enhanced efficiency of JAK2 phosphorylation, promoting cell growth/survival while numerous STAT proteins are activated by the JAK receptor [39, 40]. Furthermore, murine models have illustrated the pivotal role of STAT5 signalling in the development and maintenance of CML. One experiment utilizing retroviral transduction of *BCR-ABL1* in STAT5-knockout bone marrow failed to induce CML in recipient mice after both primary and secondary transplantation [41]. In a second model, STAT5 deletion resulted in marked depletion of *BCR-ABL1*-expressing leukaemic cells, demonstrating the importance of STAT5 in the maintenance of CML [42]. Furthermore, enhanced STAT5 expression reduced imatinib-mediated cytotoxicity in *BCR-ABL1*-positive cells, potentially linked to marked anti-apoptotic activity mediated by increased STAT5 downstream signalling [43]. Increased STAT3 levels have also correlated with imatinib resistance [44]. Regardless of the mechanism, JAK inhibitors have exhibited efficacy against *BCR-ABL1*-positive cells, overcoming TKI resistance [45]. Furthermore, the combination of the JAK-inhibitor, ruxolitinib, and nilotinib has been demonstrated to induce undetectable *BCR-ABL1* levels in patients with low-level disease [46].

2.2.2 PI3K/AKT and Autophagy

PI3K proteins communicate extra-cellular signals to modulate transcription factor activation and programming that favour cell growth/survival and inhibition of cell death. AKT is a downstream effector of PI3K and plays a major role in its signalling [47]. *BCR-ABL1* can stimulate PI3K signalling through the adapter proteins Grb2/Gab2 [48] and CBL [49] but also through loss of function of the tumour suppressor gene PTEN, which is frequently silenced in malignancy [50]. Several reports indicate that the PI3K/AKT pathway is critical for *BCR-ABL1*-induced leukaemogenesis and for CML maintenance [51] and that its interruption can circumvent *BCR-ABL1* oncogenesis [52, 53]. Another

consequence of PI3K activation is stimulation of the mTOR pathway [54], which is responsible for controlling protein synthesis, cell growth/size and autophagy.

Autophagy can occur following cell stress (i.e. loss of *BCR-ABL1* signalling) to promote cell hibernation as opposed to apoptosis, and can be reversed with restoration of the optimal environment. Recent studies have observed that whilst *BCR-ABL1* inhibits autophagy, TKI treatment restores this pathway and may contribute to molecular relapse in failed treatment-free remission attempts, despite undetectable *BCR-ABL1* levels prior to TKI discontinuation [55]. Moreover, *BCR-ABL1*-positive stem cells with knockdown of genes vital for the autophagy process failed to proliferate in an optimized environment [56]. Therefore, autophagy may be an appropriate mechanism to target for the future.

2.2.3 Ras/MEK Pathway

Activation of Ras GTPases/MEK kinases stimulates cell growth via a membrane receptor-binding cascade to activate transcription of a number of growth factor genes and is a key pathway deregulated in cancer [57]. *BCR-ABL1* activates Ras via Grb2/Gab2 phosphorylation to promote cell growth [58, 59], and persistence of Ras activity has been demonstrated in TKI-resistant CML cells [60]. Disruption of Ras signalling impairs development of *BCR-ABL1*-induced CML-like disease in mice [49, 61]. In addition, MEK inhibitors can induce apoptosis in blast crisis (BC)- and drug-resistant CML cells with targeting of CML progenitors [60, 62]. Further work is required to investigate the true potential of inhibition of this pathway in CML.

2.2.4 Src Kinases

The Src-family kinases (SFKs) are another group of widely studied downstream targets of *BCR-ABL1*. Their role is to coordinate cell growth, differentiation and motility in response to extracellular signals [63]. Initial CML cell line models showed that *BCR-ABL1* expression significantly activated the Hck and Lyn SFKs [64]. Subsequent studies demonstrated that Hck, Lyn and Fyn were required for *BCR-ABL1* cell line transformation as well as functionally phosphorylating several *BCR-ABL1* tyrosines [65, 66]. One mechanism by which SFKs contribute to disease is in assisting *BCR-ABL1* in its activation of STAT5 and AKT [67, 68]. In addition, knockdown of Lyn exhibited impressive killing of BC cells, and its upregulation in BC-CML suggested a potential role for promoting disease progression [69, 70]. However, the importance of SFKs in CML remains unclear because mouse models show that SFKs are not required for initiation of CML but, rather, support the generation of acute lymphoid leukaemia [71, 72]. The second generation TKIs, dasatinib and bosutinib, are dual Src/Abl1 inhibitors, so defining the role of SFKs in CML could have an impact on both understanding its biology and treatment [73].

2.2.5 Crkl

The adaptor protein Crkl is constitutively activated by *BCR-ABL1* [74]. Protein networks involving *BCR-ABL1* and Crkl include Cbl, STAT, PI3K, paxillin and Ras [75]. Indeed, loss of the interaction between Ckrl and *BCR-ABL1* impaired *BCR-ABL1*-induced transformation in mice [76]. The potent phosphorylation of Crkl by *BCR-ABL1* allows the measurement of the percentage of phospho-Crkl as a surrogate to *BCR-ABL1* phosphorylation levels (which are more difficult to measure) in order to experimentally examine the patient's response to TKI therapy and to predict outcome [77].

2.2.6 Long Non-coding (lnc) RNA

LncRNAs are heavily involved in normal haematopoiesis and have increasingly been implicated in haematological malignancies [78]. In CML cell line models, lncRNA-BGL3 sensitizes *BCR-ABL1*-positive cells to imatinib-induced apoptosis [79]. It also acts as a decoy for several

microRNAs that target the tumour suppressor gene PTEN, leading to its stabilisation and associated inhibition of leukaemogenesis [79]. In contrast, lncRNA-H19 facilitates leukaemogenesis in CML through upregulation of MYC, and its knockdown perturbs the pathogenicity of *BCR-ABL1* in CML cell lines [80]. Further work is required to understand the full mechanisms and impact of these lncRNAs in CML.

2.2.7 Apoptosis Deregulation

In addition to promoting cell proliferation, *BCR-ABL1* can disrupt cell death. An example of this involves a *BCR-ABL1*, Bad, BCL2 and BCL-X_L circuit (Fig. 2.2). Expression of *BCR-ABL1* can inhibit apoptosis by increasing expression of the anti-apoptotic proteins BCL2 and BCL-X_L [81]. Both STAT5 and PI3K signalling are important mediators of *BCR-ABL1*'s anti-apoptotic function. STAT5 activation by *BCR-ABL1* causes increased BCL-X_L expression [82, 83]. Furthermore, phosphorylation of the pro-apoptotic protein Bad by PI3K/Akt facilitates the interaction between the chaperone protein 14–3-3 and Bad, which restricts Bad to the cytoplasm

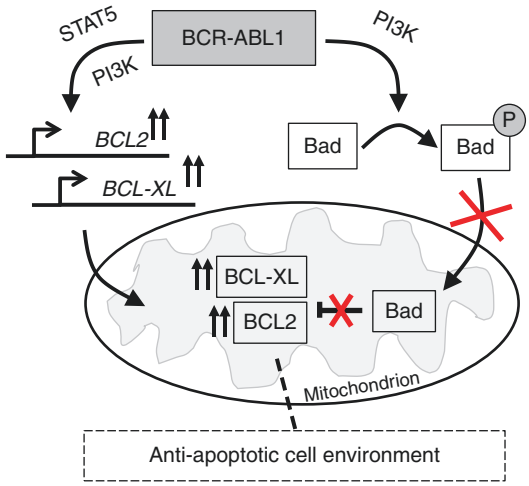

Fig. 2.2 An example of apoptotic circuitry controlled by *BCR-ABL1*. *BCR-ABL1* promotes the expression of anti-apoptotic genes BCL2 and BCL-XL and inhibits the function of pro-apoptotic protein Bad via phosphorylation (grey circle) and cytoplasmic sequestration

[84]. This prevents Bad opposing BCL2 and BCL-X_L inhibition of apoptosis in the mitochondrion.

2.3 CML Stem Cells

2.3.1 Leukaemic Stem Cells (LSCs) Are Refractory to TKIs

A seminal paper from the Holyoake laboratory showed that *BCR-ABL1* inhibition reduced LSC proliferation but failed to deplete quiescent LSCs [85]. Furthermore, LSCs have also been shown to be insensitive to more potent second-generation TKIs, despite complete silencing of *BCR-ABL1* activity [86, 87]. These studies raised the possibility of early relapse despite TKI therapy, but long-term TKI usage has rebuffed this theory [88]. Subsequent studies have strengthened the notion that survival of the LSC is independent of *BCR-ABL1* activity [89, 90]. It has also been reported that therapy-refractory LSCs exhibit a bias for *low BCR-ABL1* expression [91, 92]. Persistence of the LSCs have been postulated to be the primary causes of molecular relapse following a treatment-free remission attempt despite long-term *BCR-ABL1* negativity [88]. Several pathways have been shown to play key roles in stem cell biology (Fig. 2.3), and targeting them could lead to a promising strategy to eliminate the LSC in CML.

2.3.2 Wnt/β-Catenin Pathway

The Wnt signalling pathway has been demonstrated to be crucial for LSC self-renewal [93], and β-catenin is one of its components [94, 95]. Binding of Wnt to its receptor, Frizzled, causes disruption of ubiquitin-mediated degradation of β-catenin, freeing the molecule for nuclear translocation to activate the transcription of target genes such as the *cyclin D1* and *MYC* oncogenes [96]. *BCR-ABL1* induces aberrant PI3K/AKT signalling, resulting in upregulated β-catenin activity [53], which has also been implicated in risk of progression to BC [97]. Enhanced

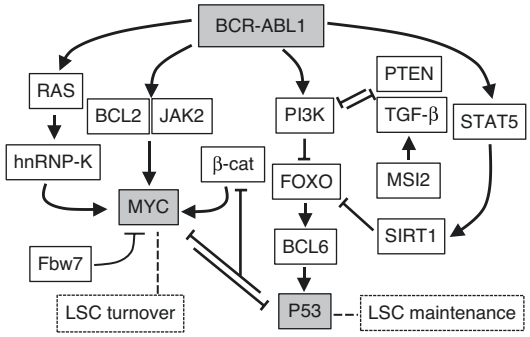

Fig. 2.3 LSC circuitry of genes discussed in this chapter. STAT5, JAK2 and PI3K all feature to control LSC-effector genes. However, quiescent stem cells have intrinsic counters to prevent potent *BCR-ABL1* signalling depleting the LSC population, such as MSI2/TGF-β, PTEN, FOXO transcription factors and Fbw7. In the context of BC-CML, the reliance on countering *BCR-ABL1* is not as important due to the incapacity of leukaemic progenitor cells to differentiate. This may explain how enhanced pathway activation (JAK2 / β-catenin) is compatible with expansion of the stem/progenitor compartment in BC. Hes1 activity enhances that of PI3K while the Hh pathway via the transmembrane receptors of PTCH and Smo regulate Gli signalling, also important for LSC maintenance

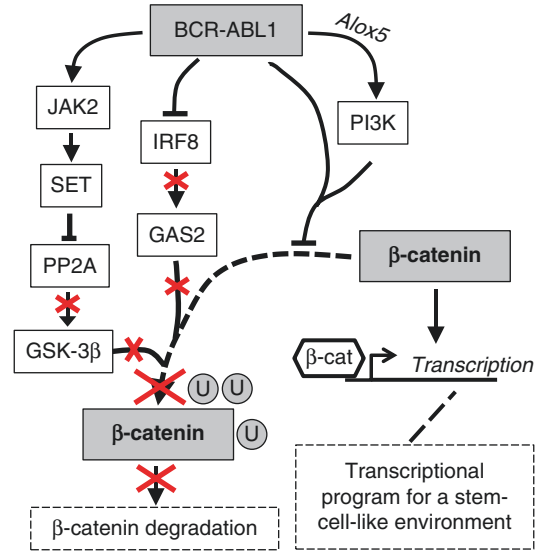

Fig. 2.4 Complex control of β-catenin in CML. *BCR-ABL1* stabilises β-catenin signalling via PI3K, JAK2 and inhibition of IRF8. Canonical stability of β-catenin is controlled by protein ubiquitination (grey circles). Thus, in CML, this pathway is activated to promote a stem-cell like environment. However, inhibition of, e.g. PP2A activation, can reverse pathogenic β-catenin signalling and synergise with *BCR-ABL1* inhibition to enhance treatment efficacy

β-catenin signalling in BC-CML confers stem-cell-like characteristics to progenitor cells leading to cellular expansion [98]. Future strategies may be to target both β-catenin and *BCR-ABL1*, as murine models have demonstrated that this approach is synergistic, delaying disease progression while depleting CML-LSCs [99] (Fig. 2.4).

2.3.3 Hedgehog (Hh) Pathway

Signalling in the Hh pathway is critical for LSC self-renewal and contributes to tissue homeostasis, regeneration and healing [100]. In *BCR-ABL1*-positive progenitor cells, increased Hh signalling is observed with marked upregulation in BC-CML [101, 102]. It also induces malignant expansions of LSCs in murine models [103]. Upregulation of Smoothened (Smo), a membrane receptor for the hedgehog ligand, has been found to augment LSCs and to drive disease progression [104]. Activation of Smo, in turn, activates Gli transcription factors, which drive expression of their downstream transcriptional targets [105]. Studies on primary CML cells found that Smo/Gli2 promoted LSC dormancy via cell cycle arrest, and an enhanced hedgehog pathway signature is observed in BC patients. Inhibition of Gli2 was able to restore LSC cycling and sensitise LSCs to TKI eradication [106]. Dual targeting with Smo inhibitors and TKIs may be a future therapeutic strategy to target both stem and progenitor cells as in vitro data suggest a reduced rate of leukaemic progression [100].

2.3.4 Notch Pathway

The Notch pathway has been demonstrated to be vital for cellular signalling and is dysregulated in multiple malignancies, including haematological cancers [107]. A member of the Notch family, Hairy enhancer of split 1 (Hes1), cooperates with *BCR-ABL1* to induce BC-CML in murine models [108]. Furthermore, over-expression of Hes1 has

been shown in BC but not CP, while dominant-negative Hes1 deterred growth of Hes1-expressing cell lines [108]. Interfering with the cross-talk between Notch signalling and *BCR-ABL1* may be achievable with combined targeting of both pathways and may be a treatment option for future exploration [109].

2.3.5 FoxO Family

BCR-ABL1 promotes deregulation of several transcription factors, including forkhead box class O (FoxO), through activation of the PI3K/Akt pathway [94]. Members of the FoxO family, in particular FoxO3a, are vital to the maintenance of LSCs [110]. *BCR-ABL1* promotes nuclear export and deactivation of these transcription factors via PI3K/Akt [111]. In mature cells, Akt signalling is strong and assists propagation of *BCR-ABL1*'s proliferative advantage. However, in LSCs, Akt signalling is inhibited by PTEN [112] and TGF-β [113]. This reverses *BCR-ABL1* inactivation of FoxO3a and allows for BCL6 transcription, which favours quiescence and self-renewal [112]. Targeting this mechanism with BCL6 or TGF-β inhibitors together with TKIs perturbed CML development and induced cell death/turnover of primitive CML cells [112, 113].

2.3.6 BCL2 Family

The proteins in the BCL2 family are key regulators of apoptosis and crucial for LSC survival [114]. BCL2 anti-apoptotic protein expression is increased in CML and is further increased in CML-BC. *BCR-ABL1* signalling also promotes CML cell survival by upregulation of anti-apoptotic BCL2 proteins, including BCL-X_L [115]. Furthermore, BCL2 acts synergistically with *BCR-ABL1* to induce BC-CML [116]. Another member of the BCL2 family, the BH3-only pro-apoptotic protein (BIM), is also downregulated in CML, supporting LSC survival [116]. TKI therapy leads to upregulation of pro-apoptotic proteins, including BIM [117]. The presence of a common synonymous variant in the BH3 functional domain of BIM has been associated with imatinib resistance and inferior molecular target achievement [118]. Selective inhibition of BCL-2 through combined therapy with venetoclax (a novel BCL2 inhibitor primarily utilized in chronic lymphocytic leukaemia) and TKI has been demonstrated to target the LSC in a *BCR-ABL1* transgenic mouse model, potentially offering a long-term cure in CML [114].

2.3.7 PP2A-JAK2-SET

BCR-ABL1 was reported to circumvent the requirement for JAK2 in its activation of STAT5 [119], but there are data demonstrating a role for JAK2 within the LSC compartment. A network involving PP2A/JAK2/Set/GSK-3β was shown to play a critical role in LSC survival [120]. Central to this pathway is PP2A, a tyrosine phosphatase whose activity is impaired in CML. 'Active' PP2A has the ability to silence key pathways which are activated by *BCR-ABL1*, including *BCR-ABL1* itself [121]. In CML-LSCs, *BCR-ABL1*/JAK2 signalling overcomes PP2A activity by enhancing the activity of SET, a PP2A-inhibitor. Blocking the PP2A inhibitory role of SET restores PP2A function and impairs the self-renewal and survival of CML-LSCs but not normal haematopoietic stem cells (HSCs) [120]. A major mechanism by which PP2A activation affects LSC maintenance is thought to be the loss of β-catenin signalling via GSK-3β mediated ubiquitination. This is coupled with PP2A silencing of *BCR-ABL1* to allow for LSC turnover and reduced leukaemic potential.

2.3.8 Bone Marrow Microenvironment

HSCs reside in the bone marrow, which provides an environment that controls haemopoiesis by coordinating HSC renewal and differentiation into functional blood cells. The bone marrow supportive environment comprises the osteoblast and vascular niches [122, 123]. The former

promotes self-renewal and quiescence, while the vascular niche is permissive of differentiation into progenitor and subsequent functional cells. In CML, it is thought that the osteoblast niche nurtures LSCs, which may explain why LSCs do not require *BCR-ABL1* kinase activity to survive TKI exposure [124, 125]. This may also contribute to BC. Since progenitor cells attain stem cell like properties, a progenitor-contingent may retreat towards the osteoblast niche for protection against TKIs, whilst retaining cycling properties that allow for faster accumulation of mutations (compared to LSCs) required for transformation.

2.4 Biology of Blast Crisis

The mechanism of disease evolution to BC-CML is still incompletely understood. This stage of the disease is characterised by the expansion of haemopoietic progenitors that fail to differentiate and interfere with normal haematopoiesis. These progenitor cells gain self-renewal capacity, differentiation arrest and survival properties that lead to uncontrolled proliferation, [98] exhibiting more stem-cell like characteristics compared to CP-progenitors. This is partially attributed to increased β-catenin activity [98] but also marked genomic and genetic instability [126, 127]. Extra chromosomal abnormalities are observed in approximately 80% of BC patients (e.g. Ph duplication, trisomy 8 or 19, loss of 17p) [128]. Pathogenic mutations in tumour suppressor and oncogenes have also been detected in BC-CML [129], and it is hypothesised that these additional hits contribute to the transition into BC [127, 129]. The rapid recent technological advances in next-generation sequencing has not only enabled attempts at unmasking the genomic landscape involved in BC-CML but has further highlighted the vast gaps of knowledge which yet remain.

2.4.1 *BCR-ABL1* and *CML-BC*

Inhibition of *BCR-ABL1* kinase activity effectively delays the onset of BC but does not eliminate the primitive population that establishes advanced disease. One interpretation is that *BCR-ABL1* signalling is required for transition to BC, especially since progression is rare in TKI responsive patients. A number of studies have found increased expression of *BCR-ABL1* in BC compared to CP. This was observed when comparing matched CP and BC samples (from the same patient) at both the mRNA [130–133] and protein levels [121, 130, 134]. Additionally, it has been shown that cells expressing higher amounts of *BCR-ABL1* have an increase in genomic instability as well as perturbed differentiation, which are intrinsic properties of BC-CML [127, 135]. These findings imply more than a passenger role for *BCR-ABL1* in BC-transformation.

2.4.2 DNA Damage/Repair

BCR-ABL1 has been shown to facilitate genomic instability via disrupting DNA repair pathways, generating reactive oxygen species and inhibiting DNA-damage-induced apoptosis, all of which may lead to retention of genomic mutations [136–140]. These events are in part tied to the level of *BCR-ABL1* expression [141]. CML CD34+ cells express high levels of *BCR-ABL1* as compared to mature cells [132], and they are highly susceptible to genomic instability compared to their healthy counterparts [89]. Although not formally shown, it is reasonable to suggest that *BCR-ABL1* provides progenitor cells with the genomic plasticity required for malignant transformation [127, 142, 143].

2.4.3 *C/EBPα* and *hnRNP-E2*

Required for myeloid differentiation [144], C/EBPα expression is reduced in cell lines expressing *BCR-ABL1* [145]. These lines responded poorly to growth-factor-induced differentiation [135], but ectopic expression of C/EBPα and *BCR-ABL1* kinase inhibition were able to reverse this differentiation block [145]. Further experiments revealed that *BCR-ABL1* negatively regulates the expression of C/EBPα via upregulation of hnRNP-E2, an RNA-binding protein which

inhibits C/EBPα expression [135]. Interestingly, analysis of CML-patient cells found that loss of C/EBPα and expression of hnRNP-E2 was restricted to BC [135]. In addition, hnRNP-E2 upregulation and C/EBPα downregulation were directly proportional to increasing levels of *BCR-ABL1* [135]. To add extra complexity to this pathway, it was recently shown that the microRNA miR-328 acts in a non-canonical way to block hnRNP-E2 regulation of C/EBPα and promotes myeloid differentiation [146]. The expression of miR-328 negatively correlates with *BCR-ABL1* expression levels and is thus downregulated in BC [146]. These experiments provide evidence of a sophisticated circuit by which enhanced *BCR-ABL1* expression can facilitate a switch to BC by disrupting myeloid differentiation.

2.4.4 Important Pathways Involved in BC-CML

2.4.4.1 MYC

The MYC proto-oncogene was one of the first genes implicated in CML disease progression. MYC is a transcription factor which governs the expression of genes enabling cell growth and proliferation and, thus, commonly activated in cancer [147]. It was originally observed that patients with BC exhibited higher levels of MYC compared to CP patients [148]. This was followed by reports that ABL1 expression enhances MYC expression and that MYC is required for *BCR-ABL1*-induced transformation [149, 150]. Although excess MYC can induce apoptosis [151], early cell line models show that *BCR-ABL1* activation of BCL2 can inhibit MYC apoptotic activity whilst retaining its proliferative advantage [152]. This is one of many examples by which *BCR-ABL1* creates 'a perfect storm' to promote leukaemogenesis.

BCR-ABL1 can control MYC expression via PI3K, JAK2 and the transcription factor E2F1 [51, 153–155], while maintaining protein stability via MEK and hnRNP-K [156]. A CML mouse model demonstrated that MYC expression is required for CML maintenance and progression. It also showed that high levels of MYC are harmful for LSCs, and ubiquitination (degradation) of MYC by ubiquitin ligase Fbw7 keeps MYC levels in check in LSCs [157]. This provides a rationale for the constrained *BCR-ABL1* kinase activity observed in quiescent LSCs [120] and selection of low *BCR-ABL1* expression in TKI-refractive LSCs [91, 92] (suggesting that enhanced *BCR-ABL1* signalling is toxic for quiescent cells). These findings, coupled with MYC's established role in myeloid differentiation [158], present MYC deregulation as a strong candidate for driving BC-transformation in CML.

2.4.4.2 p53

The normal function of p53 is to respond to cell stress events, where it becomes activated and drives transcription of genes that decide cell fate (apoptosis, DNA repair, cell cycle arrest or senescence) [159]. Early genetic studies observed inactivating mutations of p53 in approximately 20% of CML patients who progressed to BC [160, 161]. Regulation of p53 by *BCR-ABL1* is complex and unclear, with both p53 activation [162, 163] and inactivation [164, 165] being reported. However, loss or inhibition of p53 promotes BC-like disease in mice [165–167], and stabilisation of p53 in BC cells induces apoptosis [167, 168]. It has also been shown that MYC over-expression is only toxic to LSCs if p53 is present [157].

2.4.4.3 XPO1

The nuclear export protein, XPO1, is another novel candidate for the regulation of BC. Its expression is enhanced in BC patients, and pharmacological blockade of its function was shown as sufficient to kill both CP and BC-primary CD34+ cells [169]. Inhibition of XPO1 in *BCR-ABL1*-positive cell lines demonstrated that impaired nuclear transport could explain XPO1-inhibition lethality. For example, both SET and p53 were abnormally enriched in the nucleus leading to their inactivation [169]. Additional experiments revealed that long-term XPO1 inhibition caused *BCR-ABL1* degradation (via loss of SET control of PP2A activity) whereas short-term inhibition shutdown STAT5, AKT and MEK signalling prior to affecting *BCR-ABL1* activity

[169]. This suggests that both *BCR-ABL1*-dependent and -independent cell death results through XPO1 inhibition. Remarkably, an XPO1 inhibitor reversed CML symptoms (WBC count/splenomegaly) in a patient with disease progression and who was resistant to TKI therapy, highlighting a potential strategy to treat advanced disease [169].

2.4.4.4 SIRT1

Expression of SIRT1 is enhanced in CML and is, in part, regulated by *BCR-ABL1*/STAT5 [170]. This protein-deacetylase has been linked to CML BC due to its disruption of LSC turnover and DNA repair. SIRT1 suppression of p53/FoxO-controlled LSC maintenance is believed to prolong the survival of CML LSCs [170, 171]. In contrast, knockout or inhibition of SIRT1 impairs CML development and disease progression in mice by reducing proliferative and self-renewal capacity of LSCs [170, 171]. SIRT1 regulation of the DNA repair protein Ku70 in CML cell lines causes enhancement of less faithful non-homologous end joining DNA repair, which enhances mutations [172]. The knowledge that SIRT1 provides a route for LSC survival and genomic instability—the key drivers of BC-CML—offers strong evidence that SIRT1 has a major role in BC development.

2.4.4.5 ADAR1

ADAR1 is an RNA editor whose enzymatic activity converts adenosine to inosine in RNA, resulting in these nucleotides being interpreted as guanine in the ribosome, thus altering RNA behaviour and protein amino acid composition. Analysis of ADAR1 expression in CML patients showed a marked increase in expression from CP to BC, and was correlated with *BCR-ABL1* levels [173]. The BC samples also had enhanced A to I editing and altered expression of RNA-edited genes, providing evidence that the increased expression of ADAR1 in BC had a functional effect on its downstream targets [173]. Two mouse models have been developed which successfully demonstrate the important role that ADAR1 plays in CML stem cells. Following disruption of ADAR1 expression in CML mouse models, leukaemia development, maintenance and BC onset were all impaired due to the loss of primitive leukaemic cells [174]. In contrast, ADAR1 over-expression caused myeloid progenitor expansion [173]. Moreover, specific deletion of the ADAR1's RNA-editing moiety demonstrated that RNA editing is vital for CML progenitor self-renewal [174]. It is known that the RNA-editing activity of ADAR1 is required for HSC survival [175], so it is speculated that the enhanced activity of ADAR1 in BC locks the LSCs in a primitive state.

2.4.4.6 Polycomb Repressive Complexes (PRCs) and Epigenetic Regulation

Dysregulation of PRCs have been implicated in a number of haematological malignancies, including CML [176]. Early data indicated that overexpression of BMI1, a member of PRC1, correlated with inferior survival and higher risk of BC transformation [177]. Enhanced EZH2 activity, a catalytic subunit of PRC2, has also been demonstrated as necessary for the propagation of CML [178]. More recent exploration of the BC genome indicates substantial enrichment for mutations affecting the PRCs: transcriptomic interrogation of BC progenitors demonstrated both upregulation and depletion of PRC1- and PRC2-related gene sets, respectively [179].

The impact of epigenetic reprogramming is still an emerging area of research in CML. The PRCs are heavily involved with epigenetic reprogramming in BC-CML with PRC2-driven DNA hypermethylation being responsible for arrested myeloid differentiation and loss of tumour suppressor function [179]. However, DNA methylation inhibitors have failed to produce durable responses in BC-CML [179]. Gene expression analysis of BC-cells treated with hypomethylating agents revealed failure to normalize the majority of the gene expression changes associated with DNA-methylation, indicating additional layers of unidentified epigenetic regulation [179]. However, in vitro combinatorial therapy with directed inhibition of BMI1 and hypomethylating agents reduced colony formation in BC-CML cell lines by approximately 90% [179].

2.4.4.7 Mutational Landscape

While *BCR-ABL1* alone is sufficient to induce CP-CML, it is unlikely to be the sole event in more advanced stages of the disease. *BCR-ABL1* has been associated with substantial genetic instability [180], assisting in the acquisition of additional mutational events that could trigger progression to BC. In order to identify putative BC driver genes, Giotopoulos et al. utilised an impressive mouse model. The experiment centred on a transposable cassette array in the presence or absence of *BCR-ABL1* [181]. Transposition of the cassettes can either activate or deactivate the genes in proximity to the genomic insertion site. Gene activation is achieved by a transposition event within the 5′ region of the gene due to enhancer/promoter sequences in the cassette [181]. Conversely, intragenic transposition can disrupt genes causing loss-of-function. Mice with a *BCR-ABL1* only genetic background succumbed to a CML-CP phenotype, whilst 85% of the *BCR-ABL1*/transposon mice exhibited CML-BC, 5% CP and 10% AP-like disease [181]. Microarray gene expression analysis of the mice showed clustering within disease type and inter-type separation, identifying several genes known to be involved in in the development of BC. Transposition events within the BC sample cohort included STAT5, XPO1, PTEN, MYC-target genes and JAK1 [181].

The current era has been characterized by dramatic technological advances in next-generation sequencing which have enabled the identification of somatic mutational profiles that characterize various haematological malignancies, influencing diagnosis, treatment and prognosis [182–184]. In BC-CML, most patients have been identified to harbour additional mutational events in known cancer genes [185, 186] seen in up to 95% of patients in one study [185]. Mutations in RUNX1, ASXL1 and IKZF1 exon deletions are the most frequently observed events [187] while single nucleotides, insertions, deletions, fusions and aberrant splicing in multiple different cancer-related genes have all been described in BC-CML. Aberrant RAG-mediated recombination has also been demonstrated to contribute to structural rearrangements in lymphoid BC [188]. A novel class of variant, termed ‘Ph-associated rearrangements’, involving gene rearrangements and novel fusions on the chromosome arms involved with the inciting Ph-translocation, has also been observed in poor outcome patients at the time of diagnosis, including those progressing to BC-CML [185]. Moreover, the Ph-associated rearrangements were more frequently identified in patients progressing to lymphoid BC [185]. While there are minimal data regarding this novel group of mutations, their presence may highlight a cohort of patients with increased genetic instability and, therefore, increased propensity for adverse outcomes. Kinase domain mutations can be identified in approximately 50% of patients in BC-CML [185], more frequently in lymphoid BC [185]. However, these are rarely the sole event [185], frequently co-occurring with IKZF1 variants. Additionally, cancer-gene variants often pre-date the development of kinase domain mutations in approximately 60% of patients, emphasizing the genomic instability associated with the acquisition of cancer-gene mutations [185].

2.5 Concluding Remarks

The biology of CML is centred on *BCR-ABL1*’s constitutive kinase activity, which is sufficient to cause the clinical features of CP. The ability to readily model CML in both cell lines and mice has allowed for a large accumulation of knowledge regarding the molecular network of CML. These studies have shown that *BCR-ABL1* is implicated in altering almost every process within the cell to drive CML pathogenesis. This extends to dampening its own excessive signalling in LSCs, which would be otherwise unfavourable. Current literature has shown that STAT5 stands out as a vital component of *BCR-ABL1*’s induction of CML as demonstrated by two conditional knockout models [41, 189]. The investigation of primitive CML-cell biology has benefitted from the utilisation of new and powerful techniques to identify a number of important genes within this compartment. The best studied

are p53, MYC and β-catenin, which have prominent roles in both stem cell biology and BC transformation.

The link between LSCs and BC and treatment response has put the LSC and progenitor populations at the forefront of CML biology. Of particular interest is the finding that LSCs do not rely on *BCR-ABL1* kinase activity for survival. It is unknown if another protein domain of *BCR-ABL1* confers LSC survival properties. Another possibility is that *BCR-ABL1* can program LSCs in such a way that its kinase activity is no longer required. It is unknown whether the HSC or progenitor compartment gives rise to the clones responsible for BC-CML. Pinpointing the latter is important because each of these compartments has discrete biological properties and, thus, requires alternative therapeutic strategies.

Next-generation sequencing and powerful experimental modelling tools will no doubt provide a flood of information regarding CML biology as well as highlight the potential drivers of disease progression. These advances are likely to generate evidence of recurrent mutations and epigenetic marks that favour or hinder CML pathogenesis or response to treatment.

In the proteomics field, improved methods to study proteins and more powerful mass spectrometers have the potential to uncover post-translational modifications and protein interactomes. The study of proteome networks is relatively untapped in CML (although elegant examples do exist [190, 191]), making this an attractive area of interest to improve the knowledge of CML biology. The same can be said of non-coding RNA (ncRNA). It is known that deregulation of these molecules occurs in CML, for example in CP versus BC, and in primitive cells versus granulocytes [192, 193]. However, most functional work is limited to a single microRNA and target. Further work is required to understand the global ncRNA circuitry in key areas within this disease. These fields of interest are bolstered with the emerging accessibility to high-powered fluorescence microscopy, which can monitor the spatiotemporal behaviour of proteins and RNA.

Finally, availability of pathway inhibitors and genome editing (including crispR) systems [194] are powerful options to functionally validate pathways identified by genomic and proteomic studies in both cell lines and mouse models. These technologies will make for an exciting time to uncover novel mechanisms behind CML pathogenesis and the potential for translation to other diseases.

Conflict of Interest The authors declare that they have no conflict of interest for the writing of this chapter.

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