Cancer Drug Discovery and Development

Rakesh Kumar *Editor*

Nuclear Signaling Pathways and Targeting Transcription in Cancer

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Preface

Regulatory nuclear pathways feeding into the transcription of cancer-relevant molecules have emerged as the next frontier in pathway-centered cancer therapeutics. The significance of nuclear signaling in cancer is also evident by the convergence of a large number of signal transduction pathways continuously sensing extracellular milieu. The cumulative outcome of deregulated cytoplasmic and nuclear signaling is to provide a favorable environment for a cancer cell to survive by overriding death signals, to sustain an excessive hyper-mitogenic activity, to feed into deregulated cell cycle progression, and to support a defective segregation of genetic material during mitosis leading to genomic instability, to name a few essential hallmarks of cancer progression. Among other processes, chromatin remodeling and epigenetic modifications are two important nuclear regulatory arms of transcription that have offered a battery of exciting therapeutic opportunities in terms of specificity by focusing on specific modification or modifications of histone or nonhistone proteins, domain-targeting, enzymatic activities such as histone deacetylases, histone acetyltransferases, histone demethylases, or splicing factors – as all of these activities are widely deregulated in multiple human cancers. A large body of work during the last decade has demonstrated that these are targetable areas of translational cancer medicine, and therefore, a large number of small molecules or agents targeting these biological processes are rapidly moving through the preclinical development pipeline to clinical studies.

Another compelling aspect of nuclear signaling in cancer therapeutics is its inherent role in controlling the transcription of cancer-promoting factors. As many of the target gene products of nuclear signaling have functional relevance in the cytoplasmic compartment, these molecules are also at the center of signaling cascades in the cytoplasm and influenced by extracellular environment. Interestingly, the barrier of cytoplasmic and nuclear signaling is broken by recent advances in the area of nuclear receptor tyrosine kinases, which were earlier thought to be limited to the cytoplasm. Another recent excitement in the field stems from the translational control of the elements of metabolism, such as lipogenesis. In addition to therapeutic value, many of the pathways and molecules described above are also becoming

prognostic biomarkers to monitor the progression of cancer or to assess the therapeutic efficacy of a given targeted therapy. Since nuclear signaling represents the funneling junction of upstream signaling and because it imparts selectivity at the level of transcription, the overall goal of this book is to enhance our understanding of relevant processes in an integrated manner in the context of cancer medicine. The editor felt it timely to compile this book, written by the leading authorities in the field and contributing to an emerging therapeutic discipline of signaling control of transcription in cancer medicine.

Washington, DC, USA Rakesh Kumar

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Part I
Gene Regulation and Cancer

Chapter 1
Steroid Receptor Coactivators (SRCs) as Integrators of Multiple Signaling Pathways in Cancer Progression

Weiwen Long and Bert W. O'Malley

Abstract Steroid receptor coactivators (SRCs), including SRC-1, SRC-2, and SRC-3, mediate transcriptional activities of nuclear receptors and other transcription factors. SRCs' activities and functions are regulated by multiple signaling pathways, including those of hormones, growth factors, and cytokines, and are determined by post-translational modifications, including phosphorylation, ubiquitination, sumoylation, acetylation, and methylation. SRCs integrate signals from a variety of pathways that regulate multiple cellular processes such as metabolism, reproduction, and growth. For the growth response, they regulate proliferation, survival, migration, and invasion, and promote tumor development and metastasis. SRCs are highly disregulated in many types of cancers at multiple levels including gene amplification, mutation, and mRNA/protein overexpression. Alterations of SRCs are frequently associated with advanced tumor progression and drug resistance. As such, SRCs are important prognostic cancer biomarkers and could serve as therapeutic targets for cancer therapy.

Keywords Steroid receptor coactivators (SRCs) • Nuclear receptor • Gene transcription • Posttranslational modifications • Signaling pathways • Tumorigenesis • Cancer metastasis • Drug targeting

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1.1 Introduction

The p160 steroid receptor coactivator (SRC) family, consisting of SRC-1, SRC-2, and SRC-3, were originally identified as transcriptional coactivators of nuclear hormone receptors (NRs) for estrogen, progesterone, and androgen. SRC-1, also known as nuclear receptor coactivator 1(NCOA1), was cloned and characterized as the first NR coactivator in 1995 [1]. SRC-2, also known as NCOA2, GRIP1 (glucocorticoid receptor interacting protein 1), and TIF2 (transcriptional intermediary factor 2), was identified soon after the cloning of SRC-1 [2, 3]. SRC-3 was then identified by several laboratories nearly in the same year of 1997, and was provided with different names [4–7]: AIB1 (amplified in breast cancer 1), p/CIP (p300/CBP interacting protein), RAC3 (RAR-associated coactivator 3), ACTR (activator of thyroid and retinoic acid receptor), and TRAM1 (thyroid receptor activator molecule 1). Since the discovery of the first coactivator (SRC-1) in 1995, a substantial number of studies have been conducted to elucidate the molecular actions of SRCs in normal physiology and pathology. In this chapter, we will focus on the cancer-related functions of SRCs and the underlying molecular mechanisms, thereby highlighting the molecular structures and functional interacting partners of SRCs, the regulation of SRCs' activities by posttranslational modifications (PTMs), and the integration of multiple oncogenic signaling pathways by SRCs that promote tumor development and progression.

1.2 Structures and Transcriptional Interacting Partners of SRCs

SRC proteins share a common structure that contains five functional domains/regions (Fig. 1.1): the N-terminal basic helix-loop-helix-Per/ARNT/Sim (bHLH/PAS) domain, the serine/threonine rich (S/T) domain, the nuclear receptor interacting domain (RID), the CBP(cAMP-response element binding protein-binding protein) interacting domain (CID) or activation domain 1 (AD1), and the activation domain 2 (AD2) or the histone acetyltransferase (HAT) domain at the C-terminus [10, 11]. Each domain has different interacting partners that confer various functions (Fig. 1.1). The bHLH/PAS domain harbors nuclear localization signals (NLS) and is the most conserved region. This domain is also termed activation domain 3 (AD3) as it is responsible for the interaction of SRCs with multiple co-coactivators and non-NR transcriptional factors. CoCoA was shown to interact with SRC-2 through the bHLH/PAS domain and work cooperatively with p300/CBP co-coactivators to regulate NR-mediated gene transcription [12]. hBrm-associated factor 57 (BAF57), a core component of SNI/SWF chromatin remodeling complex, binds to the bHLH/PAS domain of SRCs and bridges the SNI/SWF chromatin remodeling complex to ER/SRCs transcription complex to promote estrogen-responsive gene transcription [13]. Melanoma antigen gene protein-A11 (MAGE-11) interacts with both TIF2 and AR and potentiates AR transcriptional activity probably through stabilizing the AR-TIF2 transcription complex on the target gene

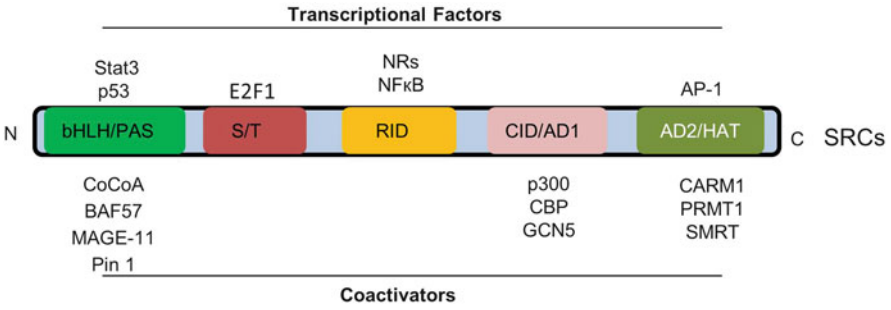

Fig. 1.1 Structural domains and transcriptional interacting partners of SRCs. SRC proteins contain five functional domains: the N-terminal bHLH/PAS domain, the serine/threonine rich (*S/T*) domain, the nuclear receptor interacting domain (*RID*), the p300/CBP interacting domain (*CID*) or activation domain 1 (*AD1*), and the C-terminal activation domain 2 (*AD2*). SRC-1 and SRC-3 harbor a histone acetyltransferase domain (*HAT*) in the C-terminus. A representative list of transcriptional interacting partners within each domain of SRCs are indicated above (for interacting transcriptional factors) or below (for interacting coactivators) the structure. Interacting proteins are referenced from [8, 9] and as mentioned in the text

promoter [14]. The bHLH/PAS domain also mediates the interaction of SRCs with several non-NR transcription factors such as Stat3 [15] and p53 [16] that are important factors in cancer.

The S/T-rich domains of SRCs are frequently targeted by protein kinases and phosphatases, which regulate SRC protein stability and activity [17]. The S/T-rich domain of SRC-3 mediates its interaction with E2F1, an essential transcription factor in cell cycle control [18]. SRCs bind to NRs through the RID domain that harbors three "LXXLL" NR-binding motifs where "L" represents leucine residue and "X" denotes any amino acid [19, 20]. The interactions of SRCs with NRs are either hormone-dependent or hormone-independent based upon the NRs that SRCs are bound to and the growth conditions. Besides mediating the interaction with NRs, the RID domain of SRC-3 is important for its interaction with NFκB [21]. Following the binding to NRs, SRCs recruit p300/CBP histone acetyltransferases through the CID domain, which promotes chromatin remodeling and the recruitment of general transcription machinery [5, 22–24]. In the C-terminus of SRCs resides the activation domain 2 (AD2) that recruits CARM1 and PRMT1 methyltransferases [25, 26]. Interestingly, the C-terminus of SRC-1 and SRC-3 also contains a HAT domain [6, 27], but its functional substrates remain to be substantiated. In addition, the C-terminus was shown to mediate the interaction of SRCs with AP-1 transcription factors that play critical roles in cancer cell proliferation and invasion [28].

Of note, SRCs also have been shown to interact with other oncogenic transcription factors such as Rb [29] and HIF1α [30], and to potentiate activities of these transcription factors, although it is unclear which precise domains within SRCs are required for these interactions. Taken together, SRCs interact with a variety of transcriptional factors and coregulators through their five functional domains, suggesting that SRCs are important molecules that integrate diverse cellular processes.

1.3 Molecular Codes of SRCs: PTMs Targeted by Multiple Signaling Pathways

As coactivators of a variety of transcription factors, the activity and functions of SRCs are regulated by multiple signaling pathways. The molecular regulation of SRCs' activity and functions are determined by post-translational modifications (PTMs, Figs. 1.2 and 1.3), including phosphorylation, ubiquitination, sumoylation, acetylation, and methylation, all of which coordinately regulate SRCs' cellular localization, stability, and the interactions with their functional partners.

1.4 Phosphorylations

SRCs are phosphorylated by protein kinases in response to multiple signals including hormones, growth factors, and cytokines. These signals work independently or in concert to regulate SRCs' activities and functions.

1.5 Hormone-Induced Phosphorylations of SRCs

Hormones stimulate target gene transcription not only by activating hormone receptors via direct binding, but also by activating protein kinases that subsequently phosphorylate hormone receptors and coregulators including SRCs (Fig. 1.4). Hormones

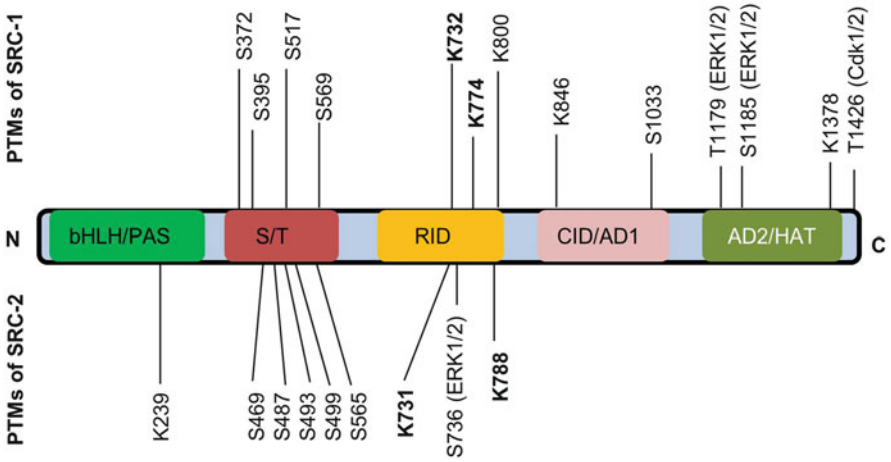

Fig. 1.2 PTMs of SRC-1 and SRC-2. Some identified serine (*S*) and threonine (*T*) phosphorylation sites and sumoylated lysine (*K*) residues of SRC-1 and SRC-2 are indicated in the schematic structure. In the "( )" are shown certain kinases that target the specific phosphorylation residues. The two conserved sumoylation sites within RID domain of SRCs are shown in *bold*

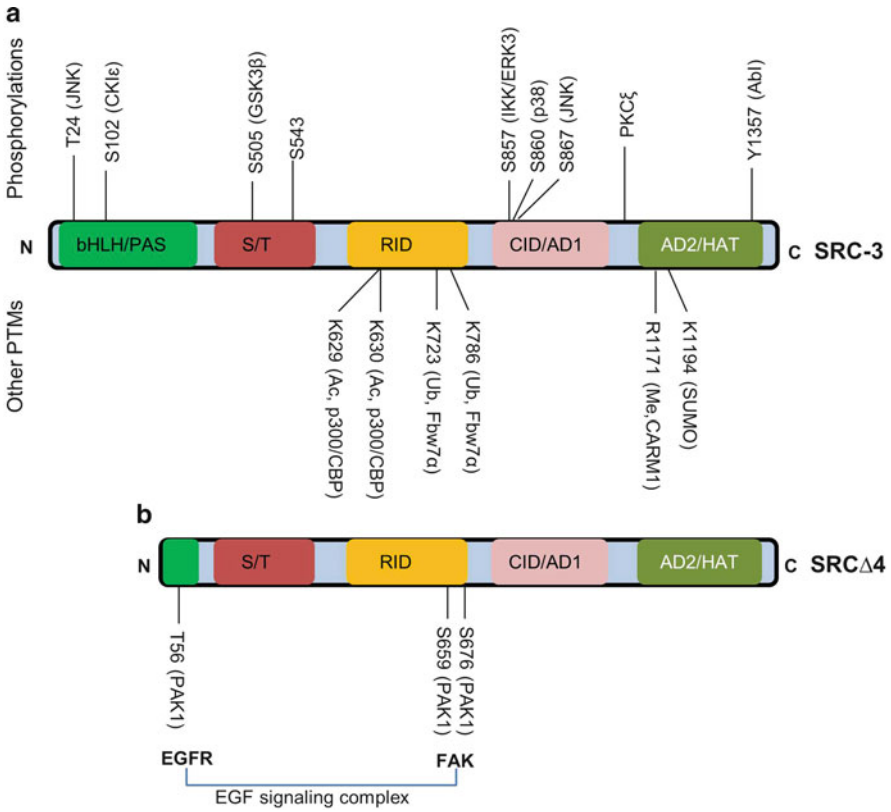

Fig. 1.3 PTMs of SRC-3 and SRC-3Δ4. (**a**) Selectedphosphorylation sites of serine (*S*), threonine (*T*), or tyrosine (*Y*), lysine (*K*) residues with ubiquitination (*Ub*), sumoylation (*SUMO*), or acetylation (*Ac*), and arginine (*R*) residue with methylation (*Me*) of SRC-3 are indicated in the schematic structures. In the "( )" are also shown certain modifying enzymes for each specific PTM. Both K723 and K786 are sumoylation sites as well that are conserved in SRCs. (**b**) Phosphorylation codes of SRC-3Δ4 that are targeted by PAK1 for EGF signal transduction to FAK. PAK1 phosphorylates SRC-3Δ4 at T56, which mediates the interaction with EGFR, and at S569 and S676, which is important for the interaction with FAK. See the text for details (It should be noted that SRC-3 contains over 50 different PTMs that have been identified by a variety of techniques)

such as estrogen, progesterone, androgen, and glucocorticoid stimulate the activation of multiple kinases such as ERK1/2, Akt, p38, and JNK; rapid activations of these kinases by hormones are referred to as non-genomic signaling in contrast to direct actions of the receptors on the nuclear genome [31]. In response to E2 stimulation, SRC-3 is phosphorylated at multiple residues including T24 in the N-terminus, S505 and S543 in the S/T-rich region, and S857, S860, and S867 in the RID region [32]. E2-induced phosphorylation of SRC-3 occurs acutely (within minutes) and is dependent on ERα [33]. Phosphorylations at these residues promote the interaction of SRC-3 with ERα and CBP, and augment SRC-3's transcriptional activity.

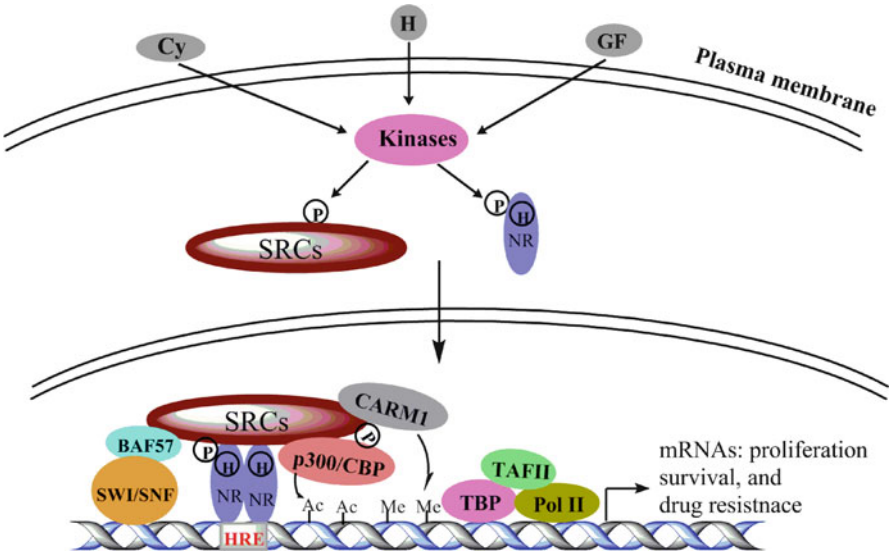

Fig. 1.4 SRCs-mediated hormone signaling and the cross-talk with growth factor and cytokine signals in regulating NR target gene expression. Upon the binding of hormone (*H*), nuclear receptors (*NRs*) dimerize and bind to the hormone responsive element (*HRE*) of target genes. SRCs coactivate gene transcription by interacting with DNA-bound NRs and then recruiting secondary coactivators including p300/CBP histone acetyltransferases and CARM1/PRMT1 histone methyltranseferases. p300/CBP and CARM1/PRMT1 elicit histone acetylation (*Ac*) and methylation (*Me*), respectively, both of which facilitates the assembly of the general transcriptional machinery consisting of TBP, TAFIIs, and Pol II, and the subsequent gene transcription. SRCs also are capable of recruiting SWI/SNF chromatin remodeling complex via BAF57 and further potentiate gene transcription. The transcriptional activities of SRCs and NRs are regulated by multiple signaling pathways including those of cytokines (*Cy*), growth factors (*GF*), and non-genomic hormone actions, mainly through protein kinase-mediated phosphorylations (*P*) that are important for the interactions of SRCs with NRs and other coactivators

Interestingly, while androgen/AR induces SRC-3 phosphorylations similar to E2/ ERα, progesterone/PR is unable to do so, suggesting there is ligand/receptor/coactivator specificity in hormone-induced SRC phosphorylations. In agreement with this notion, SRC-2 is a primary coactivator for glucocorticoid receptor (GR) and is phosphorylated upon the stimulation of dexamethason eat five residues (S469, S487, S493, S499, and S565) in the S/T-rich region and one residue (S736) in the RID region [34]. Phosphorylations of SRC-2 facilitate GR transcriptional activity. Similarly, progesterone/PR stimulates SRC-1 phosphorylations that are important for SRC-1 transcription activity (Weiwen Long and Bert O'Malley, unpublished data). Collectively, these findings demonstrate that phosphorylations of SRCs serve as an integrating link between non-genomic and genomic actions of hormones.

1.6 Phosphorylations of SRCs Induced by Cytokines and Growth Factors

SRCs also are phosphorylated upon stimulation by growth factors and/or cytokines, which facilitates their functions in coactivating NR-mediated gene expression in both ligand-dependent and ligand-independent mechanisms; these stimulations serve as important molecular mechanisms for anti-hormone resistance during cancer therapy. SRC-1 is phosphorylated at T1179 and S1185 by MAPK upon stimulation by interleukin 6 (IL-6)and co-activates AR in a ligand-independent manner in prostate cancer cells [35]. Interestingly IL-4promotes PP2A-directed dephosphorylation of SRC-1 which is important for SRC-1/Stat6-regulated IL-4 target gene expression in Ramos B lymphoma cells [36]. Cytokines such as TNFa stimulate IKK-directed phosphorylation of SRC-3 which potentiates NFκB-mediated gene expression in breast cancer cells [32, 37].

SRC-1, SRC-2, and SRC-3 all are known to be regulated by growth factor signals. Epidermal growth factor (EGF) stimulates ERK2-directed phosphorylations of SRC-1 that are important for the interaction of SRC-1 with CBP in PR-dependent transactivation [38]. T1426 in AD2 of SRC-1 is targeted by Cdk1 and Cdk2, and this phosphorylation is important for PR/SRC-1-mediated cell cycle control [39]. SRC-2 is phosphorylated at S736 by EGF stimulation, and S736-phosphorylated SRC-2 promotes AR-dependent but ligand-independent transactivation, suggesting a potential role of SRC-2-mediated cross-talk between growth factor signaling and AR signaling in recurrent prostate cancer progression [40]. SRC-3 activity is tightly regulated by growth factor signaling. Both EGF and IGF-1 stimulate SRC-3 phosphorylation at tyrosine 1357 (Y1357) that is directed by AbI kinase in breast cancer and lung cancer cells [41]. Phosphorylation of Y1357 is critical for SRC-3 oncogenic activity in coactivating ERα and NFκB and promoting anchorage-independent cancer cell growth. Heregulin 1β, an EGF-like growth factor, stimulates SRC-3 phosphorylation through ERBB2 oncogenic kinase [42] which is implicated in the breast cancer tamoxifen resistance. cAMP/PKA signaling also induces SRC-1 and SRC-2 phosphorylation and potentiates NR-mediated transactivation in a ligand-independent manner [43, 44].

1.7 Phosphorylation Codes for SRC-3Δ4 Acting as an EGF Signaling Adaptor

SRC-3Δ4 is a splicing variant of SRC-3 with the deletion of exon 4 (Fig. 1.3b) [45, 46]. In comparison with full-length SRC-3 protein, SRC-3Δ4 lacks an N-terminal bHLH/PAS region that harbors the NLS. Consequently, SRC-3Δ4 primarily localizes in the cell cytosol due to its absence of an NLS. Upon EGF stimulation, PAK1 phosphorylates SRC-3Δ4 on T56 at the N-terminus and S659 and S676 within the RID region. Phosphorylations of SRC-3Δ4 promote its localization to the plasma membrane region where it interacts with EGFR through the N-terminus, which is

mediated by T56 phosphorylation, and with FAK through the RID region, which is mediated by phosphorylations of S659 and S676(Fig. 1.3b). SRC-3Δ4 mediates the interaction between EGFR and FAK, thereby promoting EGF-induced c-Src activation and FAK phosphorylation on Y925, which in turn drives cancer cell migration and metastasis.

1.8 Ubiquitination and Its Regulation by Phosphorylation

Ubiquitination plays an essential role in regulating the stability and functions of SRC proteins. Ubiquitination of SRCs is frequently dependent on phosphorylations of specific residues that mediate the interaction of SRCs with the ubiquitin E3 ligases. The stability and activity of SRC-3 have been shown to be regulated by several protein kinase signals that are coupled to different E3 ligase complexes. GSK3β phosphorylates SRC-3 at S505 in the S/T-rich region, which is required for the binding of Fbw7α, a component of the SCF (Skp, Cullin, F-box protein containing) E3 ligase complex. SRC-3 is then ubiquitinated by $SCF^{Fbw7\alpha}$ at lysine 723 (K723) and K786 that are within the receptor-interacting domain. Interestingly, mono-ubiquitinations at K723 and K786 enhance the interaction of SRC-3 with ERα, ERα's phosphorylation at S118, and expression of the target genes in response to E2 stimulation [47]. Subsequent poly-ubiquitinations then lead to the degradation of SRC-3 and termination of SRC-3/ERα-regulated gene transcription. Ubiquitination-coupled activation of SRC-3 is also manifested by retinoic acid (RA) signaling, which involves the phosphorylation of SRC-3 at S860 by RA-activated p38, the subsequent phospho-S860-mediated recruitment of Cullin 3-based E3 ligase, ubiquitination of SRC-3, and finally activation of SRC-3/RAR-regulated gene transcription [48, 49]. In addition, SRC-3 protein stability and activity are regulated by a phospho-dependent degron that is located in the N-terminal bHLH/PAS region [50, 51]. Ser102 in the degron is phosphorylated by CKI (casein kinase I), which is required for the recruitment of a speckle-type POZ protein (SPOP)-based E3 ligase complex and the subsequent ubiquitination and turnover of SRC-3. Interestingly, phosphatases PP2A and PP1 target phosphorylated Ser102 and inhibit SRC-3 protein ubiquitination [50].

In addition to ubiquitin-dependent proteasomal degradation pathways, SRC-3 protein turnover is regulated by an ubiquitin-independent mechanism [52]. Importantly, PKCζ phosphorylates multiple residues in an acidic fragment that is important for the interaction of SRC-3 with the C8 subunit of the 20S proteasome, and enhances SRC-3 protein stability by inhibiting both ubiquitin-dependent and ubiquitin-independent proteolytic pathways [53].

In contrast with the extensive study on SRC-3, much less is known about the regulation of ubiquitination and stability of SRC-1 and SRC-2. However, a single nucleotide polymorphism (SNP) P1272S was shown to increase SRC-1 protein stability by disrupting a potential GSK3β-directed phospho-dependent degradation

code in the AD2 region of SRC-1 [54]. Although no specific phosphorylation and ubiquitination sites were revealed, an ubiquitination-coupled activation mechanism for SRC-2 was shown to be regulated by cAMP/PKA signaling [44].

1.9 Sumoylation

Sumoylation is a type of PTM that involves an addition of small ubiquitin-like modifier (SUMO) to the lysine residues of proteins [55]. In spite of its structural and enzymatic processing similarities to ubiquitination, sumoylation often alters a protein's binding affinity with the associating partners or its subcellular localization rather than degradation. All of the SRCs were shown to be sumoylated on two conserved lysine residues of the nuclear receptor-interacting domains (Figs. 1.2 and 1.3); sumoylations alter the interaction of SRCs with NRs and their transcriptional activities [56]. However, while sumoylations enhance the interaction of SRC-1 and SRC-2 with PR and AR respectively [57, 58], and their retention and transactivity in the nucleus, SRC-3 transactivity is attenuated by sumoylations at K723 and K786 [47]. As aforementioned, K723 and K786 of SRC-3 are ubiquitination sites as well, and the ubiquitinations on these sites are important for the interaction of SRC-3 with ERα and their transactivity. As such, the attenuation of SRC-3 transactivity by sumoylations at K723 and K786 is likely due to the competitive blocking of ubiquitinations on these two sites. Although the regulation of SRCs' sumoylations by phosphorylations have not been reported to date, both SRC-2 and SRC-3 harbor a phosphorylation-dependent sumoylation motif (PDSF) ψKxExxSP (where ψ is a large hydrophobic residue)that has been characterized in heat shock factor 1 (HSF-1) and MEF2A transcription factors [59, 60]. S736 within the PDSF of SRC-2 is a phosphorylation site targeted by EGF/ERK1/2 signaling [40], and K731 is a sumoylation site that enhances SRC-2 transactivity [58]. It would be of interest to determine whether this is a *bona fide* PDSF that plays a role in SRC-2 activity.

1.10 Acetylation and Methylation

SRCs act as bridging factors to recruit histone acetyltransferases such as p300/CBP and histone methyltransferases such as CARM1/PRMT1 to DNA-bound NRs to remodel chromatin and regulate gene transcription (Fig. 1.4). Interestingly, these histone modifying enzymes target not only histones but also SRCs and NRs [61–64]. SRCs were shown to be acetylated by p300 [61] and methylated by CARM1 upon E2 stimulation [63, 64]. While histone acetylation and methylation facilitate the assembly of the transcription machinery and subsequent gene transcription, acetylation or methylation of SRC-3 leads to the dissociation of NRs/cofactors complex and the termination of gene transcription.

1.11 Molecular Actions of SRCs in Cancer Cells In Vitro and in Mouse Tumor Models In Vivo: SRCs as Integrators of Multiple Signaling Pathways

SRCs, in particular SRC-3, function as important mediators and integrators of a variety of oncogenic signaling (e.g., hormones, growth factors, and cytokines) pathways to regulate virtually every aspect of cellular processes: proliferation, survival, migration, and invasion (Figs. 1.4 and 1.5). Hormone signaling acts independently

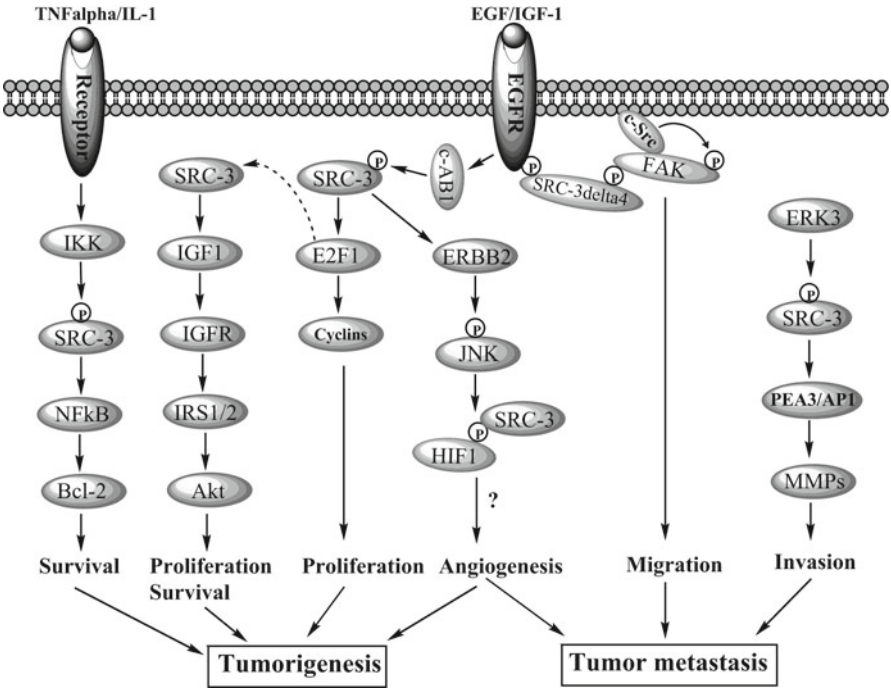

Fig. 1.5 SRC-3 integrates multiple signaling pathways to promote tumorigenesis and metastasis. Besides its regulation of hormone signaling as illustrated in Fig. 1.4, SRC-3 integrates multiple growth factor and cytokine signaling pathways to regulate a variety of cancer cell processes. Only a subset of these pathways are illustrated in Fig 1.5. In response to TNFα/IL-1 signals, SRC-3 is phosphorylated by IKKs and coactivates NFκB-mediated Bcl-2 expression for cancer cell survival. SRC-3 upregulates the expression of multiple components of IGF1-IGFR-PI3K/Akt pathway that is important for both cancer cell proliferation and survival. Upon EGF or IGF-1 stimulation, SRC-3 is targeted by protein kinases including c-AB1, and then coactivates E2F1-mediated expression of cell cycle genes including cyclins E and A. SRC-3 itself is a target gene of E2F-1 (indicated by a *dashed arrow*), and upregulation of SRC-3 by E2F1 might boost other signaling pathways regulated by SRC-3, for example, the IGF1/Akt pathway. SRC-3 is also important for the activation (phosphorylations) of ERBB2 and EGFR and the downstream kinases such as JNK, and plays a role in tumor angiogenesis, likely by coactivating HIF1. In addition, SRC-3Δ4 acts as an EGF signaling adaptor by bridging EGFR to FAK, and promotes EGF-induced FAK phosphorylations and cancer cell migration. Furthermore, SRC-3 is targeted by ERK3 kinase and coactivates PEA-3/AP-1-mediated MMP gene expression for promoting cell invasion

or synergistically with growth factor or cytokine signaling to regulate these cellular processes in which SRCs play important, integrating roles (Fig. 1.4). The actions of SRCs are exquisitely regulated by PTMs (mainly phosphorylations) that are stimulated by the oncogenic signals. Increased growth factor and/or cytokine signaling hijack SRCs to drive the progression of cancer cells from hormone-dependent to hormone-independent growth and elicit anti-hormone resistance, for example, anti-estrogen resistance in breast cancer. Based on the expression status of hormone receptors (mainly ER and PR) and growth factor tyrosine kinase receptors (mainly ERBB2 and EGFR) and the origin of cancer cells, breast cancers can be classified as 4 subtypes [65, 66]: luminal A (ER^+ PR^+ERBB2^-), luminal B (ER^+ PR^+ERBB2^+), ERBB2-enriched ($ER^-PR^-ERBB2^{++}$), and basal-like (also known as triple-negative, $ER^-PR^-ERBB2^-$). Accompanied by enhanced growth factor signaling, luminal A subtype can progress to luminal B and further to ERBB2-enriched subtype, which leads to advanced cancer phenotype and increased anti-estrogen resistance. Basal-like breast cancer cells often have upregulated EGFR expression and high aggressiveness. As discussed below, SRC-1 and SRC-3 play critical functions in all four subtypes of breast cancer, whereas the roles of SRC-2 are minor.

1.12 SRCs with Hormones/NRs-Mediated Signaling

1.12.1 Estrogen/ER Signaling in Breast Cancer

Both SRC-1 and SRC-3 act as coactivators of ERα to mediate estradiol signaling in promoting breast cancer cell proliferation and survival. SRC-1 is important for estradiol-induced cell proliferation ofMCF-7, a breast cancer cell line of luminal A subtype [67–69]. Depletion of SRC-1 differentially affected E2-inducible genes: with a significant decrease in the expression of pS2 and stromal cell-derived factor 1 (SDF-1) but little effect on c-Myc [69]. Both MCF-7 and T47D (another breast cancer cell line of luminal A subtype) overexpress SRC-3. Depletion of SRC-3in these cells diminishes the expression of estrogen/ER target genes including cyclin D1, c-Myc, and Bcl-2, and inhibits cell proliferation but increases apoptosis [70, 71]. Consequently, depletion of SRC-3 in MCF-7 cells inhibits estrogen-induced colony formation in soft agar and xenograft tumor growth in nude mice [72]. E2 exerts non-genomic signaling by activating multiple kinases including ERK1/2 and IKKα. E2-induced phosphorylations of SRC-3 via ERK1/2 and IKKα are critical for SRC-3's activity in promoting the expression of E2 target genes such as cyclin D1 and c-Myc [32, 73]. The functional relationship between SRC-3 and ERα in tumorigenesis was revealed in a mouse mammary tumor virus (MMTV)-SRC-3 transgenic mouse model [74]. Transgenic overexpression of SRC-3 induced tumors primarily in mammary glands but also in other organs including uterus and lung, suggesting SRC-3 is a *bona fide* oncogene. Ovariectomy in MMTV-SRC-3 mice greatly decreased mammary tumor formation, and genetic deletion of ERα in MMTV-SRC-3 mice by crossing with ERα-null mice completely abolished mammary tumor formation [75]. Taken together, these findings suggest that SRC-3 and possibly SRC-1 are critical for E2/ER signaling in promoting breast cancer progression.

1.12.2 Androgen/AR Signaling in Prostate Cancer

While estrogen/ER signaling is critical for the progression of breast cancer, androgen/AR signaling plays an essential role in hormone responsive prostate cancer. All of three SRC members have been shown to coactivate AR to meditate androgen signaling in prostate cancer cells. Depletion of either SRC-1 [76], SRC-2 [77, 78], or SRC-3 [79, 80] significantly decreases androgen-dependent AR transcriptional activity and LNCaP prostate cancer cell growth in culture and in xenograft tumor mouse models. On the contrary, overexpression of SRC-3 or SRC-2 in LNCap greatly enhances the responsiveness of AR to androgen or to ligand-independent stimulation and promotes cell growth.

1.13 Interplay of SRCs with ERBB2 Signaling: Anti-estrogen Resistance in Breast Cancer

ERBB2, a member of the EGFR family, is an oncogenic protein that is frequently overexpressed in advanced breast cancer. ERBB2 overexpression promotes tumor progression, and is highly associated with the progression of ER-positive breast cancer cells from estrogen-dependent to estrogen-independent growth; the overexpression is concomitant with the gain of resistance to anti-estrogen drugs [81]. Both SRC-1 and SRC-3, in concert with ERBB2, play a critical role during these processes. Genetic loss of SRC-3 completely suppressed MMTV-ERBB2 induced mouse mammary tumor formation in association with a remarkable decrease in phosphorylations of ERBB2 and the downstream kinases JNK and Akt [82]. Interestingly, tumor angiogenesis also was curtailed significantly due to the loss of SRC-3. SRC-3 was confirmed as a pro-angiogenic factor recently [83] and acts as a coactivator of HIF1α [30]. While loss of SRC-1 did not significantly decrease the average tumor number formed in MMTV-ERBB2 transgenic mice, it increased tumor latency and greatly inhibited tumor metastasis to the lung [84]. Under cell culture conditions, stable exogenous expression of ERBB2 in MCF-7 elicits an estrogen-independent cell growth and resistance to tamoxifen, both of which were significantly inhibited by depletion of SRC-3 [42]. A similar synergistic role of ERBB2 with SRC-3 or SRC-1 was shown in BT474, a luminal-B subtype of breast cancer cell line with overexpression of ERBB2 [85, 86]. ERBB2 overexpression activates the downstream kinases ERK1/2, JNK, and Akt that phosphorylate ERα and SRCs, which leads to activation of ERα under low concentration or even in the absence of estrogen [42, 87]. In addition to ERα, SRC-3 and SRC-1 interact with and coactivate other transcription factors such as Ets and PEA3 to promote cancer cell growth and invasion in response to enhanced ERBB2 signaling [88, 89]. Conversely, SRC-3 positively regulates ERBB2 expression by competing with PAX-2 (a repressor of ERBB2 gene expression) for the binding to the ERα-bound site of the ERBB2 gene [90]. These findings suggest a positive feedback between ERBB2 and SRC-3 that regulates breast cancer cell proliferation and anti-estrogen resistance.

1.14 Interplay Between SRCs and EGFR Signaling: From the Membrane to the Nucleus

EGF/EGFR signaling is implicated in multiple cancers including those of breast, prostate, and lung. EGF signaling stimulates SRCs' phosphorylation and activation; activated SRCs then work with transcriptional factors including E2F-1, ETS, and AP-1 to regulate cell proliferation, migration, and invasion (Fig. 1.5).

In breast cancer, increased EGFR signaling is frequently associated with the invasive and triple-negative phenotype. In a triple negative breast cancer cell line MDA-MB231, SRC-3 and its N-terminus-deleted isoform SRC-3Δ4 elegantly regulate distinct aspects of the EGF signaling at different cellular locations (Fig. 1.5). SRC-3Δ4 acts as a signaling adaptor bridging EGFR to FAK at the plasma membrane to mediate activation of FAK and promote cell migration upon the EGF signal [46]. SRC-3 is phosphorylated by Ab1 kinase atY1357 in response to EGF stimulation, and this phosphorylation is important for SRC-3's function in coactivating AP-1 and E2F1 in the nucleus and promoting cell growth [41]. Intriguingly, EGFR tyrosine phosphorylation and activity is regulated by SRC-3. Depletion of SRC-3 greatly decreased EGF-induced EGFR tyrosine phosphorylation, which led to decreased JNK kinase activity and growth inhibition of MDAMB231 cells [91]. While the detailed molecular mechanism is unclear, downregulation of tyrosine phosphatases by SRC-3 partly contributes to an increase of EGFR phosphorylation.

In prostate cancer, enhanced EGFR signaling is highly associated with castration-resistant (androgen-independent) cancer progression. On one hand, EGF signaling stimulates AR tyrosine phosphorylation (Y534) via c-Src kinase [92], and AR tyrosine phosphorylation promotes its nuclear localization and transactivity. On the other hand, EGF signaling targets SRCs via MAPK-directed phosphorylations. For example, SRC-2 is phosphorylated at S736 by EGF stimulation, and S736-phosphorylated SRC-2 promotes AR-dependent but androgen-independent transactivation [40]. Together, EGF signaling stimulates AR/SRCs' transactivity and promotes prostate cancer cell growth in culture and tumor growth in castrated mice [40, 92].

1.15 Regulation of IGF-1/Akt Signaling by SRC-3 and SRC-1

IGF-1/Akt signaling is another molecular pathway that is regulated by SRCs and is critical for SRCs' oncogenic functions (Fig. 1.5). The IGF-1/Akt signaling pathway initiates with the binding of IGF-1 to its receptor (IGF-1R) on the cell membrane and the subsequent activation of IGF-1R by auto-tyrosine phosphorylation, followed by the recruitment of insulin receptor substrate (IRS) proteins and IRS-mediated activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway [93]. SRC-3 regulates the expression levels of multiple components of the IGF-1/Akt pathway, includingIGF-1/IGF-2, IGF-1R, IRS-1/IRS-2, and Akt in breast cancer cells [94] and prostate cancer cells [95], thereby regulating the activities/

phosphorylations of IGF-1R and Akt and promoting cancer cell proliferation and anchorage-independent cell growth. The regulation of IGF-1/Akt signaling by SRC-3 was corroborated in vivo. Increased IGF-1 expression and activation of IGF-1R, Akt, and mTOR was seen in MMTV-AIB1 (SRC-3) transgenic mice, which led to spontaneous mammary tumor formation [74]. In contrast, genetic depletion of SRC-3 in v-Ha-Ras transgenic mice caused a remarkable decrease in tumor initiation and metastasis to the lung, partly due to decreased expression of IRS-1 and IRS-2 and the attenuated Akt activity [96]. Loss of SRC-1 did not affect primary mammary tumor growth, but greatly decreased tumor metastasis to the lung in MMTV-polyoma middle T antigen (PyMT) transgenic mice by downregulating ERBB2 expression and Akt phosphorylation [97].

1.16 Interplay of SRCs with Cytokine Signaling in Promoting Cancer Cell Aggressiveness

IL-6 signaling promotes castration-resistant prostate cancer progression by activating AR in an androgen-independent mechanism [98–100]. Besides the direct effect on AR, IL-6 signaling modulates SRCs' activity as well. SRC-1 phosphorylation by MAPK in response to IL-6 is important for ligand-independent activation of AR [35]. In addition, upregulation of SRC-2 in LNCaP cells upon long-term treatment with IL-6 is associated with acquired resistance to bicalutamide, an anti-androgen drug [101]. Conversely, cytokine production can be regulated by SRCs. For example, SRC-1 upregulates colony-stimulating factor-1 (CSF-1) expression and promotes the recruitment of macrophages to the tumor site, which contributes to tumor metastasis [97].

SRC-3 is phosphorylated upon the stimulation of cytokines such as TNFα and IL-1β [32, 37]. Phosphorylations of SRC-3 stimulate the interactions of SRC-3 with ERα and NFκB, which are important for TNFα-induced cyclin D1 expression and cell proliferation [102]. Interestingly, SRC-3 was shown to interact with translational repressors TIA1 (T cell restricted antigen 1) and TIAR (TIA1 related homologue) and regulate the translation of TNFα and interlukin 1 mRNAs [103], for which the implication in cancer is unclear at present.

A recent study revealed an intriguing interplay between a cleaved isoform of SRC-1 and TNFα signaling during endometriosis [104] which shed new light on the roles of SRCs in inflammation-associated diseases such as cancers. Endometriosis is an inflammation-driven disease that is initiated by the migration of endometrial cells to distal sites. While TNFα is well-known as a critical driving factor for endometriosis, it is less clear how the intrinsic pro-apoptotic activity of TNFα is silenced during this process. It is shown in this interesting study that TNFα-induced MMP9 cleaves SRC-1 at Pro-790, and the c-terminus of cleaved SRC-1 promotes endometriosis by blocking caspase8-mediated apoptosis and by stimulating

epithelial-to-mesenchymal transition (EMT) for increased invasiveness. Given the positive associations of both SRC-1 and inflammation with advanced and metastatic tumor stages, cytokine signaling and SRC-1 conceivably could synergistically promote cancer progression and metastasis following similar mechanisms as shown in endometriosis. Indeed, SRC-1 was shown to positively regulate TWIST, a master regulator of EMT, thereby promoting tumor cell migration/invasion and metastasis [89]. It would be interesting to determine whether SRC-1 undergoes proteolytic cleavage to produce the cleaved isoform during cancer progression and whether this cleaved form of SRC-1 is responsible for the upregulation of TWIST and EMT-associated tumor cell migration and invasion.

1.17 Phospho-dependent Regulation of SRC-3 by an Atypic MAPK for Cancer Cell Invasion

SRC-3 promotes cancer cell invasion by coactivating PEA-3- and AP-1-regulated matrix metalloproteinase (MMP) expression [105–107], but the invasive signals to SRC-3 and the molecular regulation of SRC-3 proinvasive activity were not elucidated until a recent study that revealed a phospho-dependent regulation of SRC-3 proinvasive activity by an atypical MAPK ERK3 (Fig. 1.5) [108]. ERK3 was identified as an interacting partner of SRC-3 by immunoprecipitation-mass spectrometry (IP-MS) analyses. ERK3 phosphorylates SRC-3 at serine 857 (S857), and this ERK3-mediated phosphorylation at S857 is essential for SRC-3's interaction with the ETS transcription factor PEA3, promoting upregulation of matrix metalloproteinase (MMP) gene expression and proinvasive activity in lung cancer cells. ERK3/SRC-3 signaling drives cancer cells to invade and form tumors in the lung. As such, this study not only revealed a molecular mechanism for regulating SRC-3 proinvasive activity, but also identified a novel oncogenic function for ERK3 in promoting lung cancer invasiveness.

1.18 Alterations of SRCs and the Clinical Implications in Cancers

As transcriptional coactivators integrating multiple signaling pathways to regulate cancer cell proliferation, survival, migration, and invasion, SRCs are highly dysregulated in a variety of cancers including breast cancer, prostate cancer, endometrial cancer, ovarian cancer, lung cancer, colorectal cancer, liver cancer, and pancreatic cancer. Alterations of SRCs, including primarily gene amplification, mRNA or protein overexpression, are implicated in cancer progression and metastasis and tumor resistance to therapeutic interventions.

1.19 Gene Amplification

Gene amplification is one of the fundamental features for defining an oncogene. SRC genes, in particular SRC-3 (also known as AIB1-Amplified in breast cancer gene 1), are amplified in multiple human cancers. Gain of copy numbers of SRC-1 and SRC-2 genes were reported in a recent study with a cohort of 218 prostate tumors consisting of 181 primaries and 37 metastases [109]. Of particular note, SRC-2 gene amplification was detected in 8 % of primary tumors and 37 % of metastases. Gain of SRC-2 expression is associated with increased rates of prostate tumor recurrence. SRC-3 gene amplification has been shown in multiple cancers including breast cancer (with 5–10 % frequency [4, 110]), ovarian cancer (with 7–25 % frequency [110, 111]), colorectal cancer (with 10–32 % frequency [112, 113], lung cancer (with 8.2–27 % frequency [114, 115]), and hepatocellular cancer (40 % frequency, [116]). SRC-3 gene amplification contributes to upregulation of SRC-3 mRNA and protein in cancers, and is positively correlated with advanced tumor stages.

1.20 Mutations

In contrast to high frequencies of gene amplification and mRNA/protein overexpression, gene mutation of SRCs is rarely detected in cancers. Even though a few point mutations of SRC-1 [117] and SRC-2 [109] were identified in tumors, the frequency is very low (~1 %) and the pathological association is undetermined. Interestingly, a fusion between MOZ (monocytic leukemia zinc finger) gene and TIF2 (SRC-2) gene has been repeatedly detected in acute myeloid leukemia [118–120]. MOZ-TIF2 fusion protein retains the PHD zinc finger domain and the MYST domain of MOZ and the CBP-interacting domain (CID) and activation domain 2 (AD2) of TIF2. The recruitment of p300/CBP via the CID of MOZ-TIF2 is essential for leukemogenesis of the fusion gene [120].

1.21 Upregulation of mRNA and Proteins

Expression of SRCs, in particular SRC-3 and SRC-1, are frequently upregulated in a variety of cancers. SRC-3 is the second most overexpressed oncogene among all human cancers, second only to c-myc. Upregulation of SRCs is often co-current with elevated protein kinase signaling, which indicates high tumor grade, increased tumor invasiveness, and tumor resistance to therapeutic treatments. A substantial number of studies have investigated the expression and clinical implication of SRCs in a variety of cancers, with focus mostly on breast cancer and prostate cancer.

1.22 Breast Cancer

Breast cancer progression relies on two major signaling pathways: hormone (e.g. E2/ERα) signaling and epidermal growth factor signaling pathways [81]. Antihormonal drugs (e.g. tamoxifen, an estradiol antagonist, and letrozole, an anti-aromatase inhibitor) have been commonly used for treating ER/PR-positive breast cancers. However, antihormone resistance (either naïve or acquired resistance after therapy) and associated cancer recurrence have been major obstacles for curing breast cancer patients. The acquisition of resistance to antihormonal drugs is often associated with a transition of hormone signaling-dependent to growth factor signaling-dependent tumor growth [121, 122]. SRC-1 and SRC-3 are overexpressed at high frequency in breast cancer, and their overexpression is implicated both in hormone-dependent and in hormone-independent breast cancer progression and metastasis.

SRC-1 expression has been shown to be significantly increased in around 19–30 % breast tumors [123–125]. In line with its primary role in promoting cancer cell migration and invasion in vitro and tumor metastasis in animal models, upregulation of SRC-1 is highly correlated with lymph node metastasis and poor disease-free survival (DFS) of breast cancer patients [88, 126]. Another important role for SRC-1 is the regulation of cancer cell sensitivity to anti-hormone drugs. Indeed, SRC-1 expression has been demonstrated as a predictor of anti-estrogen resistance and/or tumor recurrence following therapy [125, 126].

The implication of SRC-3 expression in breast cancer progression has been extensively studied. From various separate studies [4, 72, 124, 127, 128], overexpression of SRC-3 mRNA and protein has been shown in the range of from 13 % to 74 % of breast tumors, with an average of around 50 % overexpression rate. In agreement with the broad roles of SRC-3 in regulating cancer cell proliferation, survival, migration, and invasion in cultured cells and in animal models, overexpression of SRC-3 is positively associated with advanced tumor grade, increased tumor invasiveness and metastasis, and worse DFS in both ERα-positive and ERα-negative breast tumors [4, 87, 124, 127]. SRC-3 overexpression is frequently associated with enhanced protein kinase signaling. Tyrosine kinase receptor ERBB2 is frequently amplified (gene copy) and overexpressed (mRNA and protein) in cancers [129], with the highest frequency in breast cancer (~25 %). Simultaneous overexpression of SRC-3 and ERBB2 are reported in several studies [87, 128, 130, 131], and their co-overexpression indicates increased tumor resistance to tamoxifen treatment and increased tumor recurrence. Although the regulation of the IGF1R/Akt signaling pathway by SRC-3 has been well demonstrated by cell culture studies and mouse mammary gland tumor models, the correlation between these two has not been shown in tumor studies of breast cancer patients. Given the frequent alterations of both of these two factors in breast cancer, it is of high clinical significance to investigate the association of SRC-3 and IGF1R/Akt signaling pathways and their implications in cancer progression in more detail.

In contrast with ample evidence for the critical roles of SRC-1 and SRC-3 in breast cancer progression and metastasis, few studies have provided conclusive data to support a definite role for SRC-2 in breast cancer.

1.23 Prostate Cancer

Androgen/AR signaling plays a critical role in the initiation and progression of prostate cancer, and has been a major therapeutic target for treating this disease [132, 133]. Androgen ablation therapy (mainly by chemical castration) effectively inhibits tumor growth during the initial treatment. Unfortunately, most tumors relapse and become resistant to androgen ablation therapy. Castration-resistant cancer progression or recurrence is frequently associated with an advanced and metastatic tumor phenotype and has been a major obstacle for curing prostate cancer. AR and its target genes are commonly expressed in and are believed to drive recurrent prostate cancers [134]. In addition to AR gene mutations and AR overexpression, alterations in AR coactivators including SRCs and upregulation of growth factor signaling are two other major molecular mechanisms for castration-resistant tumor progression [135]. While genetic alterations of the SRC-1 gene are rare in prostate tumors, upregulation of SRC-1 has been shown by a few studies [76, 117, 136]. Upregulation of SRC-1 protein is associated with lymph node metastasis [76] and tumor recurrence after androgen deprivation therapy [117, 136]. Like SRC-1, SRC-2 expression is highly increased in recurrent prostate tumors following androgen deprivation therapy [117, 136]. More importantly, a recent study showed that elevated SRC-2 expression, probably due to SRC-2 gene amplification, was detected in both primary and metastatic prostate tumors [109]. Similar findings were reported for SRC-3 in prostate cancer [79, 106, 137, 138]. Upregulation of SRC-3 is positively correlated with increased Akt activity in prostate tumors [79, 138], which affirms a positive regulatory role of SRC-3 in Akt signaling in prostate cancer.

1.24 Lung Cancer

SRC-3 functions as an oncogene in lung cancer. Transgenic overexpression of SRC-3 in mice causes spontaneous lung tumor formation [74]. SRC-3 gene amplification and protein overproduction were shown in as high as 27 % of non-small cell lung cancers (NSCLCs) in one study [115]; overexpression correlates with poor disease free and overall survival. Interestingly, ERK3, a kinase that phosphorylates SRC-3 and confers SRC-3 pro-invasive activity in lung cancer cells, was shown to be highly upregulated in lung cancer [108]. Overexpression of SRC-3 protein in lung cancer also was reported in two other studies [139, 140]. The implications of SRC-1 and SRC-2 in lung cancer are not known.

In addition to the cancer types discussed above, SRC-3 has been shown to be overexpressed in many other cancers: a 64 % overexpression rate in high grade ovarian tumors [141], a 67 % overexpression rate in hepatocellular carcinomas [116], a 35 % overexpression rate in colorectal carcinomas [112], and a nearly 70 % overexpression frequency in pancreatic tumors [142].

1.25 Tumor Suppressor Functions of SRC-3 and SRC-2 in Specific Tissue Context

In contrast with the ample evidence for SRC-3′s function as an oncogene in a multitude of cancers, deletion of the SRC-3 gene promotes proliferation of lymphocytes and induces spontaneous malignant B-cell lymphoma upon aging in mice [143]. Similarly, while SRC-2 has been identified as an oncogene in prostate cancer, a recent study revealed a tumor suppressor role for SRC-2 in liver cancer [144]. Although the molecular mechanisms underlying the unexpected tumor-suppressing roles of SRC-2 and SRC-3 are not understood, these findings suggest that SRCs, as transcriptional coactivators, can regulate cell proliferation and survival either positively or negatively, depending upon the cell- and tissue context.

1.26 SRCs as Prognostic Biomarkers and Therapeutic Drug Targets

Increased expression and activities of SRCs not only are implicated in cancer progression and metastasis, but also are positively associated with drug resistance, including anti-estrogen resistance in breast cancer [87, 123, 125, 145, 146], resistance to EGFR inhibitors in lung cancer [115]), and resistance to chemotherapeutic drugs such as cisplatin and doxorubicin [30, 147, 148]. Hence, novel therapeutic drugs targeting SRCs might be utilized individually or in combination with other therapeutic drugs for treating cancers in different subtypes and at different stages.

1.27 Targeting SRCs by Intervening NR-SRC Interactions

The binding of SRCs with NRs via SRCs' 'LXXLL' NR interacting motifs is critical for their transcriptional activity. In addition, the flanking amino acids of the 'LXXLL' motif are shown to confer an order of specificity on differential NR/SRCs interactions. On the basis of these molecular mechanisms, there have been efforts to develop peptides containing LXXLL motifs or identifying small molecules that can disrupt the interactions between NRs and SRCs. A screening of the phage display

library identified peptides that specifically and effectively inhibit ERα or ERβ transactivity [149–151], but it was unclear what specific ER/SRC interaction(s)was affected by these peptides. Soon after this, a peptide specifically blocking the interaction of SRC-2 with TRβ [152] and other peptides preventing the binding of SRC-1 with ERα or ERβ [153, 154] were identified utilizing a similar strategy. In addition, small molecule inhibitors (SMIs) were identified for targeting SRCs' interaction with ERα [155] and TRβ [156]. Although these identified peptides or SMIs were shown to efficiently inhibit NRs' transactivity, their efficacy on NR/SRCs-mediated cell functions were not evaluated in animals.

1.28 Small Molecule Inhibitors Targeting SRCs for Degradation

Since overexpression of SRC proteins, in particular SRC-3, is highly associated with advanced tumor progression and drug resistance, a potentially more effective strategy would be to identify small molecules that directly target SRCs and downregulate SRCs' protein stability and activities. Based on this idea, a recent study identified a SMI for SRC-1 and SRC-3 by both activity-based and stability-based screening assays [157]. This proof-of-principle drug, gossypol, downregulates the stability and activities of both SRC-1 and SRC-3 via direct binding, but is less selective forSRC-2 and other cofactors. Importantly, gossypol greatly increases the response of cancer cells to inhibitors of growth factor signaling, including MEK inhibitor AZD6244, EGFR inhibitor AG1478, and IGF-1R inhibitor AG1024. This study demonstrates that SRCs are accessible therapeutic targets to SMIs and encourages additional high throughout screenings for identifying drugs targeting SRCs.

SRCs also can serve as diagnostic and prognostic biomarkers, as they are altered in cancers at multiple levels including gene amplification, mRNA/protein overexpression, and protein posttranslational modifications. Of particular interest is the phosphorylation of SRC-3 at S857. S857 appears to be a hotspot that is targeted by multiple kinases and confers to SRC-3 and SRC-3Δ4 a variety of oncogenic functions: augmentation of cancer cell motility when targeted by PAK-1 [46], increase of cancer cell invasion when targeted by ERK3 [108], and gain of chemotherapeutic drug resistance when targeted by IKK [30]. It is of significant clinical interest to test whether phosphorylation on this site is positively associated with human cancer progression and metastasis, thereby serving as a diagnostic and/or prognostic tumor biomarker.

1.29 Conclusion and Perspective

Cancer cells must acquire a variety of capabilities for tumor initiation, uncontrolled outgrowth, invasion of surroundings, and metastasis to the distant organs. Hanahan and Weinberg have summarized these required capabilities as eight hallmarks of

cancer: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, and two more recently recognized hallmarks-reprogramming of energy metabolism and evading immune destruction [158]. Despite little information on the roles of SRCs in those two emerging hallmarks, substantial evidence exists that SRCs act as integrators for the other hallmarks, thereby making great contributions to cancer cells for acquisition of these hallmarks. It has been recognized that cancer cells reprogram energy metabolism to provide fuels and biosynthetic intermediates (nucleosides and amino acids) for uncontrolled cell growth and/or invasion by enhancing energy consumption from glycolysis [159]. Although the roles of SRCs in reprogramming energy metabolism of cancer cells have not been revealed, a number of studies, mostly by using SRC knock-out animals, have shown that SRCs are critical regulators of energy metabolism of glucose and lipids [160–166]. SRC-1 has distinct functions in cell metabolism of white and brown adipose tissues: loss of SRC-1 renders animals more susceptible to high fat diet-induced obesity, whereas loss of SRC-2 or SRC-3 confers protection against it. As such, future work on SRCs in cancer cell metabolism is warranted and should provide new insights on the molecular mechanisms by which SRCs alter the cues for uncontrolled growth and aggressiveness of cancers.

Evading immune destruction and harnessing tumor-associated inflammation is another hallmark that is important for cancer progression. The roles of SRCs in this process have been scarce but surely are worthy of investigations. Although their exact roles in tumor-associated inflammation remain to be determined, SRCs are engaged in cytokine signaling and inflammation. As mentioned above, SRCs are activated (phosphorylated) by cytokine signaling and function as coactivators of NFκB and/or Stats to positively regulate cytokine signaling in cancer cells [32, 35, 37, 101, 102] and in animal models of inflammation-associated disease [104, 167–169]. Hence, it would be of clinical significance to determine in more detail the roles of SRCs in the underlying molecular mechanisms of tumor-associated inflammation. Finally, as our recent proof-of-principle study demonstrates that SRCs are accessible therapeutic targets for SMIs, more effort should be put on future high throughput screenings for therapeutic drugs targeting SRCs for cancers.

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Chapter 2
Role of Alteration/Deficiency in Activation (ADA) Complex in Cell Cycle, Genomic Instability and Cancer

Shakur Mohibi, Shashank Srivastava, Hamid Band, and Vimla Band

Abstract In eukaryotes, DNA wraps around histone proteins to form highly condensed chromatin structures that usually remain inert and inaccessible to proteins involved in DNA-related processes. Thus, multitudes of important DNA-related biological processes, including transcription, replication, DNA repair, apoptosis, chromosome condensation, and segregation, are dependent upon alteration of this chromatin structure so that proteins involved in these processes can access the DNA. This required change in chromatin structure is brought about by binding of various chromatin modifying proteins that loosen the chromatin by distinct mechanisms, one of which is covalent histone modification. Various histone post-translational modifications, specifically acetylation, play a major role in opening up of this highly condensed chromatin allowing access to proteins involved in the several important processes. Histone acetyl transferases (HATs) and histone deacetylases (HDACs) are important for maintaining a steady-state level of this particular post-translational modification in cells and are present in multi-subunit complexes. One such multi-subunit HAT complex is the alteration/deficiency in

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activation (ADA) complex, which was originally discovered in yeast and is now known to be also present in mammalian cells as part of much larger HAT complexes. In this chapter, we discuss various components of the ADA complex with a special focus on the adaptor proteins Ada3 and Ada2 (Ada2a and Ada2b) for their role in important physiological processes, such as the cell cycle, genomic integrity, DNA repair response, and in pathology such as cancer. Further, we discuss recent developments using various inhibitors to target the HAT enzymes and disrupt HAT complex function as an anti-cancer strategy.

Keywords Ada3 • Ada2 • HATs • Cell cycle • DNA repair • p53 • Nuclear receptors • Viral oncogenes • Cancer • HAT inhibitors

2.1 Introduction

Precisely regulated cell proliferation is essential for embryonic development as well as adult tissue homeostasis, and uncontrolled cell proliferation is a hallmark of cancer [1, 2]. Coordination of cell-cycle progression with chromosomal duplication maintains genomic stability, a critical cancer-associated trait [3]. Deregulated cell-cycle components have now also emerged as key biomarkers and therapeutic targets in cancer [4]. Thus, a better understanding of the cell-cycle machinery and its aberrations in cancer are of fundamental importance in cell and cancer biology. In eukaryotes, DNA is wrapped tightly around histone proteins to form chromatin that facilitates higher order folding of DNA [5]. This greatly limits the accessibility of DNA by various proteins involved in transcription, replication, cell division, and DNA repair [6, 7]. Post-translational modifications of histones play an important role in modifying the folding of chromatin and affect the functions involving chromatin [8]. Acetylation of histones is one of the most important and widely studied post translational modification and it has emerged as a conserved mechanism that is invariably altered in cancer [9, 10] as it plays key roles in chromatin assembly, accessibility to transcription and replication machineries, and genome stability (Fig. 2.1) [11]. Acetylation of histones loosens the chromatin structure allowing proteins involved in various processes to bind to DNA (Fig. 2.1) [8]. Steady-state levels of histone acetylation and its dynamic changes represent a balance between histone acetyl transferases (HATs) and histone deacetylases (HDACs) [12].

HATs usually function in multi-subunit complexes and are evolutionary conserved [13]. One such complex is the yeast alteration/deficiency in activation (ADA) complex that consists of the HAT general control non derepressible 5 (Gcn5, originally called Ada4), ADA HAT complex component 1 (Ahc1) and adaptor proteins Ada2 and Ada3 [14]. The ADA genes were initially discovered in yeast based on mutations in them conferring resistance to GAL4-VP16 toxicity [15–18]. These genes included *Ada1*/*Hfi1*, *Ada2*, *Ada3*, *Ada4* and *Ada5* (*Ada4* and *Ada5* are commonly referred to as *Gcn5* and *Spt20*, respectively). The ADA complex has been shown to act as a co-activator complex in yeast and is involved in transcription [19, 20].

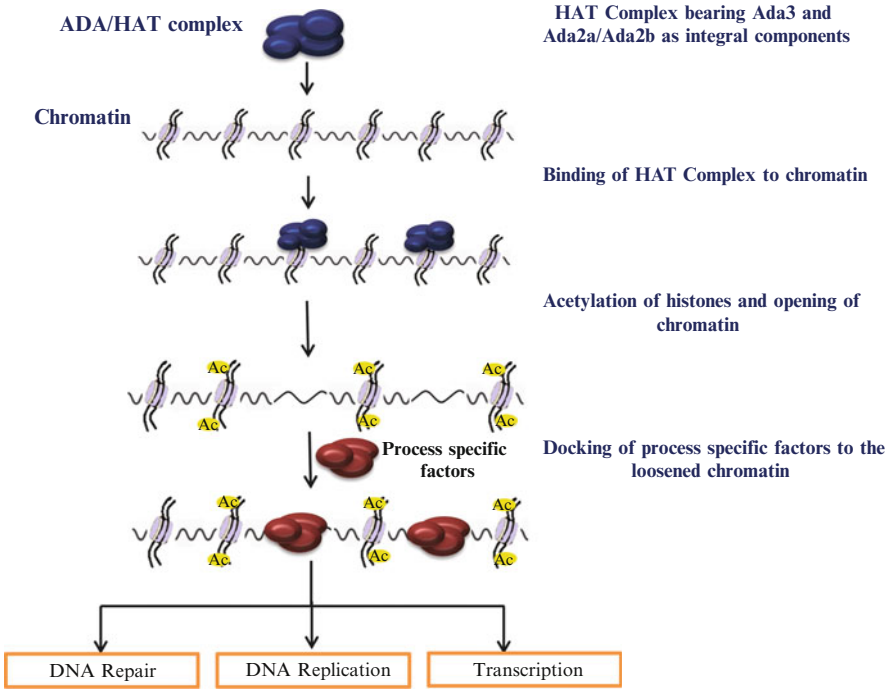

Fig. 2.1 A schematic model showing different cellular processes regulated by ADA complex or by HAT complexes, that contain Ada2a/Ada2b and Ada3 as core components, through histone acetylation and chromatin remodeling. ADA/HAT complex binds to chromatin at promoters/enhancers/DNA repair sites/origins of replication and aid in loosening up of chromatin by acetylating histones at the sites. This allows various factors involved in distinct processes such as transcription, DNA replication and DNA repair to have access to DNA and perform their functions

In addition to the ADA complex, Gcn5, Ada2 and Ada3 proteins are also a part of Spt-Ada-Gcn5 acetyltransferase (SAGA) and SAGA-like (SLIK) complexes in yeast [19–21]. These proteins and the ADA complex are highly conserved from yeast to mammals where they usually form a HAT module of large multi-functional complexes such as the Spt3/Taf9/Gcn5 acetyltransferase complex (STAGA) (human homolog of yeast SAGA complex), the Ada2a-containing complex (ATAC), and the TBP-free TAF complex (TFTC) [13, 22].

The mammalian cells are more complex and contain multiple HATs (e.g., p300, CREB-binding protein (CBP), p300/CBP-associated factor (PCAF), Tip60 and MOF) in addition to Gcn5. The mammals also contain adaptor proteins Ada3, and two different homolog proteins for yeast Ada2 – Ada2a and Ada2b – that, along with various HATs, form essential and functional module of several multi-subunit HAT complexes, as mentioned above [13, 23, 24]. Notably, *Ada2a* and *Ada2b* are two different homologs of the same yeast *Ada2* gene, and are present in separate complexes in higher eukaryotes; however, these two homologs are unable to complement each other functionally indicating that both have a distinct set of functions [25–27].

Although the main function of the ADA complex is in histone acetylation, the components of this complex have been shown to regulate functions of various non-histone proteins such as nuclear hormone receptors (e.g., estrogen receptor, retinoic acid receptor, retinoic X receptor, and androgen receptor), p53, c-myc, retinoblastoma protein (Rb), and various E2Fs [28–39]. As the role of various mammalian HAT proteins in various physiological and pathological processes is the focus of multiple reviews [11–13, 23], in this chapter we have kept our focus on Ada proteins, particularly focusing on Ada3 and Ada2 (Ada2a and Ada2b), as these adaptor proteins have been shown to be indispensible for the functions of various HATs. We will discuss their potential roles in cell cycle, genomic stability, and their disruption in pathology focusing on cancer. Given the current knowledge that adaptor proteins have no independent enzymatic function and only function as part of the HAT complex, we will also briefly discuss functions of various HATs as we go through reviewing functions of Ada2a, Ada2b, and Ada3. Lastly, as the therapeutic efficacy of relatively general HDAC inhibitors in cancers has recently gained importance [40], we will discuss current knowledge of various HAT inhibitors considering that it is likely that more-targeted agents to alter acetylation in cancer cells will provide improved anticancer strategies.

2.2 The ADA Complex, Histone Acetylation and Chromatin Remodeling

As mentioned earlier, histone acetylation plays a fundamental role in modeling of chromatin structures in order for proteins involved in important DNA-related processes to access the DNA. The primary function of the ADA complex has been shown to be transcriptional regulation of genes by modulating histone acetylation at gene promoters [19, 20]. Although yeast Gcn5 alone can acetylate free core histones in vitro, various studies in yeast have conclusively proven that Gcn5 requires both Ada2 and Ada3 for efficient acetylation of nucleosomes, both, in vitro and in vivo [19, 41, 42]. Thus, even though Ada2 and Ada3 do not possess intrinsic HAT activity, they are essential for acetylation of nucleosome histones by Gcn5. Accordingly, it has been shown that yeast Ada2, Ada3, and Gcn5 form a catalytic core of the ADA and SAGA HAT complexes, which is necessary and sufficient in vitro for nucleosomal HAT activity and lysine specificity of the intact HAT complexes [19, 42]. The yeast ADA complex has been shown to preferentially acetylate lysine residues 9, 14, and 18 (and to a lesser extent lysine 23) of histone H3; however, the yeast ADA complex is unable to acetylate histone H4 in vitro [42]. The authors further demonstrated that Ada2 enhances catalytic activity of Gcn5. Moreover, they demonstrated that Ada3 is necessary for Gcn5-dependent nucleosomal HAT activity in yeast extracts and is important for expanding the lysine specificity of the ADA complex [42]. Similar to yeast, studies with mammalian Gcn5, Ada2b (present in STAGA complex), and Ada3 proteins have shown that these proteins can form a heterotrimer in vitro and can efficiently acetylate nucleosomal

arrays [43]. Unlike yeast proteins, neither Ada2b nor Ada3 was demonstrated to enhance the acetylation of free-core histones by mammalian Gcn5 in vitro. However; efficient acetylation of chromatin by Gcn5 was shown to require both Ada2b and Ada3 [43]. Interestingly, unlike Ada2b, the Ada2a homolog of the yeast Ada2 was unable to facilitate acetylation of nucleosomal histone H3 in HAT assays in vitro, even though it could form a complex with Gcn5 and Ada3 both in vitro and in vivo [43]. Similar to earlier reports in *Drosophila*, the authors convincingly demonstrated that Ada2a and Ada2b have non-redundant functional roles in mammalian cells. Contrary to this report, which indicated that the mammalian Ada3 protein is unable to enhance HAT activity of Gcn5 on free core histones in vitro, a recent report from our laboratory demonstrated that mammalian Ada3 is able to enhance HAT activity of p300 even on free core histones [44].

Similar to important roles of these proteins in vitro for histone acetylation, several studies have shown that these proteins are important for histone acetylation in vivo. In yeast, it has been shown that depletion of Ada3 or Ada2 drastically affects the histone acetylation in vivo in cells and this has been linked to defects in replication and DNA damage repair in yeast cells (see later sections). Furthermore, *Drosophila* null for *Ada2b*, had reduced H3K9 and H3K14 acetylation during development; however, *Ada2a* deletion did not have any effect on acetylation of these residues [27]. Although, deletion of either *Ada2a* or *Ada2b* was lethal for *Drosophila* development, both proteins were shown to have non-redundant functions in *Drosophila*. Similarly, a later study demonstrated requirement of Ada3 for viability of *Drosophila* embryos [45]. These *dAda3* mutants were shown to be defective in histone acetylation at H3 K9, H3 K14, and H4 K12, whereas there were no defects observed in acetylation of H3 K18 or H4 K5, K8, or K16. These defects in histone acetylation were shown to affect the position effect variegation at certain loci and in the transcription of specific genes. Additionally, we recently demonstrated that depletion of Ada3 from mammalian cells results in drastic down-regulation of histone acetylation at various lysine residues such as H2A K5, H2B K5, H3 K9, H3 K56, and H4 K8 [44]. This dramatic down-regulation in various histone acetylations underscores the important role of the ADA complex in histone acetylation.

In order for appropriate proteins to bind DNA and carry out their function, the process of histone acetylation routinely needs to be coupled with chromatin remodeling, which occurs by nucleosome sliding leading to removal of nucleosomes at promoter regions or at DNA damage sites [46, 47]. The chromatin remodeling complex SWItch/Sucrose Non Fermentable (SWI/SNF) is an important complex that has been shown to be involved in nucleosome sliding [46, 47]. Interestingly, this complex has been shown to work in concert with HAT complexes at promoters of various genes and has been shown to be involved in gene activation [48]. More importantly, the recruitment of SWI/SNF complex onto various promoters is believed to be dependent on acetylation of nucleosomal histones by the SAGA complex [49]. Acetylated histones form a prerequisite for the recruitment of SWI/SNF complex through bromo domains present in Swi2/Snf2 [50]. Consistent with this observation, it has been shown that the SWI/SNF complex is capable of efficiently displacing

nucleosomes from chromatin that are specifically acetylated by SAGA complex compared to nucleosomes that are not acetylated [51]. Additionally, it has been shown that yeast Gcn5 directly regulates the binding of SWI/SNF complex to chromatin, through acetylation of Snf2 component of SWI/SNF complex [52]. Snf2 acetylation by Gcn5 results in the dissociation of SWI/SNF complex from acetylated histones, thus inhibiting SWI/SNF complex function [52]. Furthermore, the ATAC HAT complex in *Drosophila* has been shown to stimulate nucleosome sliding by stimulating the ISWI, SWI/SNF, and RSC chromatin remodeling complexes [53]. These findings highlight a fundamental and functional link between histone acetylating complexes and complexes involved in chromatin remodeling and also underline the importance of HAT complexes in chromatin remodeling as histone acetylation acts as a pre-requisite for chromatin modeling through nucleosome sliding.

2.3 The ADA Complex Functions as a Co-activator for Nuclear Hormone Receptor-Mediated Transcription

The ability of nuclear hormone receptors (NR) to up-regulate or down-regulate the target gene expression is determined by their association with cofactors that may fall under the category of co-activator or co-repressor [54]. When bound to a co-activator, nuclear receptors up-regulate the gene expression whereas binding of a co-repressor leads to the down-regulation of target gene expression. Over the past two decades a number of co-activators have been studied extensively by different laboratories and an important class of co-activators was identified as steroid receptor co-activators (SRC-1, -2 and -3) by Bert O'Malley's group [55]. X-ray crystallography studies have demonstrated that a typical co-activator contains α helical LXXLL binding motif (where L is leucine and X is any amino acid) referred to as NR box through which it binds to a groove on the surface of ligand binding domain of nuclear receptor [56, 57]. In addition to SRCs, several novel nuclear receptor co-regulators, such as BCAS3, PELP1, and DLC1, have also been identified and characterized [58].

In regard to the role of the ADA complex in NR activation, initial observations that the ADA complex plays a role in NR-mediated transcription came from yeast Ada3 (yAda3) [59]. Though yeasts do not have NRs, the yAda3 protein was found to be associated with exogenously expressed NRs. In this context, it was shown that the ADA complex is required for the transactivation function of the glucocorticoid receptor (GR) [59]. The investigators further demonstrated that deletion of any of the components of the ADA complex reduces the activity of the GR responsive-lacZ reporter compared to the wild type. Notably, deletion of *Ada3* was found to cause a greater reduction in this activity than deletion of either *Ada2* or *Gcn5* alone [59]. Furthermore, Ada2 was shown to enhance the activity of the GR responsive reporter in mammalian cells [59].

The yAda3 also interacts with other NRs such as ERα, RXRα, and TRα, but not with RARα [60]. Reporter assays in yeast have demonstrated that yAda3 potentiates

the AF-2 activity of ERα and RXRα when overexpressed in yeast and mammalian cells [60]. The authors further showed that, other subunits of the ADA complex, Ada2 and Gcn5, are also required for ERα- and RXRα-mediated transactivation [60]. Subsequently, our laboratory demonstrated that mammalian Ada3 interacts with ERα and RXRα and augments their transactivation and increases the levels of target gene expression [29–31]. More importantly, shRNA-mediated knockdown of *Ada3* significantly down-regulated estrogen-responsive genes and as a result suppressed ER-mediated cell proliferation, thus supporting an important role of co-activators in the NR-mediated functions [29, 30]. Subsequently, other investigators performed mutational analyses of Ada3, and observed that similar to classical NR co-activator, Ada3 binds to RAR through its LXXLL motifs [32]. In summary, these studies provide significant evidence that Ada3 functions as a co-activator in NR signaling.

2.4 Interaction of the ADA Complex with Non-Nuclear Hormone Receptor Proteins

The p53 protein is a tumor suppressor protein that transactivates stress responsive genes and regulates the cell cycle in response to DNA damage [61]. The activation domain of p53 possesses notable similarity with the activation domain of other transcriptional activators such as the activator of herpes simplex virus, VP16 [61]. Like other transcription factors, VP16 also requires co-activators for its activity, and, in yeast, the requirement of the ADA complex was first shown for VP16 transactivation [15, 16, 62]. The similarity of the p53 activation domain sequence with the activation domain of VP16 and the requirement of the ADA complex for its activator function generated the rationale to study the interaction of ADA components with p53 [63]. Investigators identified two activation subdomains (ASD-1, -2) in the p53 amino-terminus that require yeast adaptor complex Ada2/Ada3/Gcn5 for transcriptional activation [63]. ASD-1 was less dependent on the ADA complex than ASD2, and Ada3 was the most critical component in the complex for the function of p53 [63]. Subsequently, work from our laboratory, and that of other laboratories, demonstrated a direct interaction of Ada3 with p53 and its function as a co-activator for p53-mediated transactivation [34, 35, 64].

Full transcriptional activation of p53 requires its C-terminal acetylation by p300/CBP and PCAF [65, 66], and we subsequently demonstrated that Ada3 recruits p300 to acetylate p53 and regulates its transcriptional activity [35]. In this context, shRNA-mediated knockdown of Ada3 dramatically down-regulated p53 target genes. Most importantly, loss of Ada3 led to inhibition of DNA damage-induced p53 acetylation and cell-cycle arrest [35]. Subsequently, another group delineated the role of Ada2 in the function of p53 [67]. Using chromatin immunoprecipitation assay, the authors demonstrated that Ada2b, but not Ada2a, gets recruited to the p53 response element on promoters of target genes [67]. Indeed, the study revealed that Ada2a and Ada2b function in a non-redundant manner and only Ada2b is found to be the component of STAGA complex in humans [67].

In addition to NRs and p53, Ada3 also interacts with IL-1α and β-catenin [36, 68]. Typically IL-1α mediates its action in a secreted form. However, a proteolytic maturation product of IL-1α, known as IL-1α N-terminal peptide (IL-1NTP) found in the nucleus, acts as a transcription factor and is involved in variety of cellular processes such as control of cell proliferation and apoptosis [69]. A study performed in yeast and mammalian systems delineated the interaction of IL-1NTP with p300, PCAF, Gcn5, and Ada3 [68]. In yeast, the fusion protein Gal4BD/IL-1NTP was found to have a growth inhibitory effect that requires an intact SAGA complex [68]. More importantly, deletion of any of the components of SAGA complex was found to completely attenuate the suppressive effect, confirming the necessity of an intact SAGA complex for the action of IL-1NTP [68]. In the mammalian system, IL-1NTP was found to interact with p300, PCAF, Gcn5, and Ada3, and eventually integrate into the p300-PCAF complex, thus enhancing the transcriptional activation of this complex [68].

The role of the ADA complex in the activation of β-catenin, which is involved in developmental processes through the Wnt pathway, has also been demonstrated [36]. The Wnt pathway is crucial for development and proliferation, and abrogation of this pathway is linked to cancer development. Both Ada2a and Ada3 have been shown to interact directly with β-catenin and mediate its acetylation [36]. As a consequence, Ada2a and Ada3 regulate the target gene expression of β-catenin. Also, reduced levels of these proteins lead to repression in β-catenin-dependent cell proliferation [36]. Further studies in this context are warranted to conclusively address the role of the ADA complex in the Wnt-β-catenin pathway.

2.5 The ADA Complex and Cell Cycle

The eukaryotic cell cycle consists of the following four phases: G1, S, G2, and M [70]. During the G1 phase, cells accumulate nutrients, grow, and duplicate various cell organelles, except chromosomes, which occurs later [70]. Before entering the S phase, cells examine their size, determine the availability of appropriate nutrients and growth factors, and ensure that there is no DNA damage [70]. The process of DNA replication occurs during the S phase, and it provides a means for duplication of genetic material that can then be equally segregated into daughter cells during the process of mitosis [70]. The eukaryotic cell-cycle progression thus depends on proper coordination of DNA replication and segregation of duplicated chromosomes to daughter cells, a process precisely regulated by modification of chromatin that allows accessibility to factors involved in these processes. Thus, the HAT complexes involved in modulating the structure of chromatin, as mentioned earlier, play an important role in the cell-cycle progression. Consistent with this, various ADA complex components have been shown to play indispensible roles in various phases of cell cycle.

Recently, our laboratory demonstrated an important role of Ada3 in the G1-S phase transition as well as in mitotic progression of cell cycle [44]. To elucidate the physiological function of Ada3, we generated a conditional knockout mouse for the *Ada3* gene. We observed homozygous $Ada3^{FL/FL}$ mice were viable, fertile, and

exhibited no gross abnormalities compared to *Ada3*$^{FL/+}$ or *Ada3*$^{+/+}$ controls, whereas Ada3$^{-/-}$ mice were lethal at E3.5 stage [44]. The failure of *Ada3*$^{-/-}$ embryos to remain viable beyond E3.5 suggested a potential role of Ada3 in cell proliferation because extensive cellular proliferation occurs during this early stage of embryogenesis.

Subsequently, by using *Ada3* deletion in *Ada3*$^{FL/FL}$ mouse embryonic fibroblasts (MEFs) we showed that Ada3 is required for efficient cell-cycle progression through the G1 to S phase transition as well as for proper mitosis [44]. Detailed analyses in this system revealed that an Ada3-c-myc-Skp2-p27 axis controls the progression of the G1 phase to the S phase and partly contributes to cell-cycle delay upon deletion of *Ada3* [44]. Microarray analysis showed that loss of *Ada3* resulted in several changes in gene expression that were involved in mitosis [44]. Consistent with this, *Ada3* deletion led to severe mitotic defects and formation of multi-nucleated cells. Also, the transition from the G2/M phase to the G1 phase was delayed upon deletion of *Ada3* [44]. Thus, Ada3, a core component of the ADA complex, is important in G1 phase as well as in mitosis during the cell-cycle progression.

Another group also showed a role of the ATAC complex in mitosis [71], where knockdown of ATAC complex components, such as *Ada2a* and *Ada3*, led to severe mitotic defects. These defects included centrosome multiplication, defective spindle and midbody formation, generation of binucleated cells, and a slow transition from G2/M to G1 phase [71]. The mitotic defects were attributed to the inefficient acetylation of the Cyclin A/Cdk2 complex by Gcn5 due to knockdown of *Ada3* or *Ada2a* [71]. Similar to mammalian *Ada3*, deletion of *Ada3* as well as *Gcn5* in flies leads to defective H3S10 phosphorylation, an event that marks the initiation of mitosis. This suggests a role of the ADA complex in the process of mitosis in flies as well as mammals [45].

Several reports have shown the role of the ADA complex component Gcn5 in replication, which is consistent with the important role of histone acetylation in DNA replication. In yeast, it was shown that Gcn5 is required for replication-coupled nucleosome assembly [72]. *Gcn5* deletion mutants in yeast showed a reduced level of H3K56 acetylation, a mark linked to replication-coupled nucleosome assembly in yeast [72]. Similar to Gcn5, deletion mutants of *Ada3* and *Ada2* showed defects in replication suggesting an important role of these components in the replication process [72]. In mammals, Gcn5 has also been shown to play an important role in the process of replication by controlling the acetylation of Cdc6, an important replication licensing factor [73]. Although, the role of other ADA complex components in replication needs to be explored extensively, these initial reports show promising results for a role of the ADA complex in replication. Taken together, these studies unequivocally support a critical role of the ADA complex in cell-cycle progression.

2.6 Role of the ADA Complex in DNA Damage Response

In addition to metabolic and transcriptional processes, the chromatin structure plays an important role in the DNA damage response (DDR) process. The DDR is manifested by assembly of DNA damage repair proteins at the site of damage [74].

Histone modifying enzymes such as HATs along with ATP-dependent chromatin remodeling complexes allow these DNA damage proteins to access DNA at the damage sites [74]. In the context of DDR, the role of Gcn5 and Ada2 has been elucidated [75]. This study focused the role of Gcn5 and Ada2 in nucleotide excision repair of yeast *MET16*, a gene regulated by these two components of the SAGA/ADA complex [75]. The role of Gcn5 and Ada2 in nucleotide excision repair was revealed by the finding that deletion of either *Ada2* or *Gcn5* delays the cyclobutane pyrimidine dimer removal on the *MET16* locus [75]. In another study, investigators showed that the STAGA complex interacts with UV-damaged-DNA binding factors DDB1 and DDB2 and this interaction facilitates the recruitment of nucleotide excision repair machinery through HAT activity of Gcn5 [76]. Furthermore, another role of STAGA complex in p53-dependent gene activation through Gcn5 and its recruitment on *p21* and *GADD45* promoters upon UV damage was shown [67]. Besides STAGA, the TFTC HAT complex is also reported to have an important role in DDR [77]. Researchers identified SPT130 as an integral subunit of the TFTC complex. Interestingly, SPT130 possesses homology with the UV-damaged DNA binding factor [77]. Given the presence of SPT130 in TFTC, the investigators found that TFTC is recruited on UV-damaged DNA and brings about the acetylation of histone H3 on the UV-damaged site, clearly suggesting a role of TFTC in DDR [77]. The role of p300 in DDR is also documented where it has been shown to stabilize and transactivate p53 in response to DNA damage [78]. Other HATs, such as MOF, acetylate H4 K16 and mediate the recruitment of repair proteins, such as Mdc1, 53BP1, and Brca1, upon ionizing radiation-induced DNA damage [79]. Similarly, Tip60 also acetylates core histones, and inactive Tip60 has been found to be associated with late double strand breaks [80–82].

Although a fairly good number of studies have delineated the role of various HATs in DDR, the role of the ADA complex per se had not been studied until recently. We demonstrated that loss of *Ada3* leads to dramatic genomic instability as seen by various chromosomal aberrations, which were further enhanced upon DNA damage [83]. Loss of *Ada3* led to an increase in the levels of DDR proteins, such as pATM, p53BP1, pRAD51, and γH2AX [83]. Significantly, *Ada3*-null cells exhibited a delay in the disappearance of the DNA damage foci for γH2AX, 53BP1, and CtIP after ionizing radiation, suggesting the important role of Ada3 in DDR [83]. Together these findings reveal a new role of Ada3 in the DNA repair process and maintenance of genomic stability and warrant further research to determine if other components of the ADA complex also regulate genomic stability and repair foci disappearance.

2.7 The ADA Complex and Cancer

As described above, components of the ADA complex are fundamental in the cell-cycle progression, regulation of various transcriptional factors, and in maintaining genomic stability. Not surprisingly, several of the components of the ADA complex are hijacked by viruses and are known to interact with viral onco-proteins, such as

human papilloma virus 16 (HPV16) E6, simian virus 40 (SV40) large T, or adenoviral protein E1A, thus linking the ADA complex to oncogenesis [64, 84–91].

Our laboratory identified human Ada3 as a HPV16-E6-binding protein [64]; importantly, HPV16 is the most common HPV associated with human cancers. Significantly, Ada3 bound to immortalization-competent E6 mutants, and also to mutants that were incapable of binding to p53 [64]. We further demonstrated that E6 targets Ada3 for degradation, thus abrogating the function of p53 through an alternate pathway [64]. Subsequently, other investigators showed that degradation of Ada3 by E6 abrogated p14ARF-p53-mediated senescence pathway and led to E6-induced immortalization [92]. Further, p300/CBP have been shown to be associated with HPV16 E6/E7, adenoviral E1A, as well as SV40 large T antigen [84–88]. Also, the yeast SAGA complex has been shown to be important in adenoviral E1A induced growth inhibition [89, 90]. Recently, the HAT Gcn5 was shown to functionally interact with the adenoviral E1A protein [91]. Together, these studies underscore the important role of the ADA complex in viral oncogenesis.

Moreover, functions of several important cellular oncogenes (e.g., c-myc, E2Fs) and/or tumor suppressors (e.g., p53 [see above], Rb, BRCA1) have been shown to be regulated by various HATs and HAT complexes [37–39, 93–98]. c-myc oncogene was shown to be associated with TRRAP, a subunit of the Ada2b and Ada3 containing mammalian STAGA complex [93]. Upon mitogenic stimulation, c-myc, a sequence specific transcription factor, was shown to induce histone acetylation at its target gene promoters through recruitment of TRRAP [93]. More importantly, the C-terminal ATM-related domain of TRRAP has been shown to be required for c-myc driven transformation [94]. Furthermore, another study demonstrated that c-myc recruits TRRAP as well as Gcn5, by interacting with the STAGA HAT complex [95]. Both TRRAP and Gcn5 were shown to enhance the transcriptional activation of c-myc through its N-terminal activation/transformation domain [95]. Accordingly, an N-terminal deletion mutant of c-myc was unable to bind to STAGA complex and showed reduced transcription activation potential [95]. Interestingly, a similar naturally occurring truncated form of c-myc has been shown to be deficient in transforming primary cells [96]. These studies reveal an essential role of the STAGA HAT complex in c-myc induced oncogenic transformation.

E2F family of transcription factors regulate several cell cycle associated genes and have been shown to be regulated by various HATs [38, 39]. Two independent studies demonstrated that PCAF, p300 and CBP HATs bind to and acetylate E2F-1, -2 and -3 [38, 39]. This reversible acetylation of E2Fs by various HATs was shown to enhance their stability and increase their DNA binding ability as well as transactivation potential [38, 39]. Furthermore, transactivation domains of E2F1 and E2F4 were shown to directly bind and recruit Gcn5 and TRRAP, most likely as subunits of HAT complexes [97]. This study suggests that E2F transcription factors stimulate their transcriptional activation by recruiting the HAT complex components TRRAP and Gcn5, thus providing a mechanism to relieve the transcriptional repression at E2F target gene promoters [97]. Similar to E2Fs, p300 and PCAF have been shown to acetylate and regulate the function of the important cell-cycle regulator and tumor suppressor retinoblastoma (Rb) protein [37, 98]. This acetylation event has also

been shown to be essential in nuclear localization of Rb and plays an important role in cellular differentiation. Thus, de-regulation of these HATs could potentially disrupt the function of Rb and contribute to oncogenesis. Additionally, the tumor suppressor BRCA1 has been shown to bind Gcn5 and TRRAP-containing HAT complexes [99]. This interaction has been shown to be indispensible for BRCA-1 mediated transcriptional regulation as well DNA repair. These studies emphasize an essential role of HAT complexes in regulating various functions of important cellular oncogenes as well as tumor suppressors.

p300 and CBP HATs have also been shown to be involved in leukemogenesis [100]. Somatic mutations of p300 and CBP have been reported in hematological malignancies [100]. These mutations include translocations of p300 and CBP genes that result in their fusion with the monocytic leukemia zinc finger (MOZ) gene or with the mixed lineage leukemia (MLL) gene [100]. These translocations lead to the formation of MOZ-p300/CBP or MLL-p300/CBP fusion proteins that can have aberrant loss-of-function or gain-of-function properties and can play an important role in cellular transformation [100]. Also, germline mutation of CBP causes Rubinstein-Taybi syndrome, a condition that predisposes its patients to cancer [101, 102]. Interestingly, recent reports from two different laboratories show that high expression of p300 in hepatocellular carcinoma correlates with poor survival and aggressive features in HCC, such as epithelial to mesenchymal transitions [103, 104]. Although further investigation is required in this area of research, the above observations highlight the importance of HAT complex components in the regulation of oncogenesis.

Our previous findings demonstrated Ada3 is a critical component of HAT complexes that regulate ER function [30]. These findings and the observations that overexpression of other ER co-activators, such as SRC-3 predicts clinical outcomes in breast cancer patients [105, 106], prompted us to examine potential significance of Ada3 expression/localization in human breast cancer patients [107]. Using immunohistochemical analysis of Ada3 expression in breast cancer tissue specimens from a large cohort of patients with known clinico pathological parameters and survival data, we reported that predominant nuclear Ada3 expression correlated with ER expression and predicted a favorable clinical outcome while predominant Ada3 expression in the cytoplasm correlated with ErbB2/EGFR expression and poor patient survival. These studies suggest an important role of Ada3 in breast cancer progression. Further studies are needed to examine the molecular mechanism of differential localization of Ada3 (and other components of the ADA complex) in the promotion of breast oncogenesis.

2.8 New Emerging Functions of the ADA Complex

In the previous sections of the chapter, we discussed well documented functions of the ADA complex. However, several laboratories have recently demonstrated an important role of the ADA complex and its components in endoplasmic reticulum stress. Endoplasmic reticulum is a cellular organelle that is involved in proper

folding and post-translational modifications of secretory and transmembrane proteins and thus houses many chaperone proteins [108, 109]. Interestingly, endoplasmic reticulum has also been shown to act as a sensor of cellular stress [108]. Various forms of cellular stress cause an increase in the demand for protein folding, challenging the capacity of chaperones present in the endoplasmic reticulum [110–112]. This leads to accumulation of unfolded and misfolded proteins in the endoplasmic reticulum lumen that causes cells to initialize a cascade of signaling events which are collectively called the unfolded-protein response (UPR) [110–112]. Three distinct endoplasmic reticulum localized transmembrane protein sensors, inositol requiring 1α (IRE1 α), double-stranded RNA-dependent protein kinase (PKR)-like ER kinase (PERK) and activating transcription factor 6 (ATF6) initiate three different UPR signaling cascades in the cells [110–112]. The ADA complex and its components have been shown to be involved in at least two of the three UPR signaling cascades [113–117]. In this context, it was first shown in yeast that Gcn5 interacts with Ire1 protein kinase and that ADA complex is specifically required for UPR but not for heat shock protein response [113]. The authors proposed that Ire1 activation recruits the ADA complex specifically to the promoters of the genes involved in UPR [113]. In a follow-up study, the authors demonstrated that ADA complex components Gcn5, Ada2, Ada3 and Ada5 interact with Ire1 and HacI [114]. HacI is a transcription factor that is required for UPR and its translation is regulated by Ire1 [111]. HacI is constitutively expressed; however, its mRNA is not efficiently translated [111]. Activation of Ire1 leads to formation of a new processed form of HacI mRNA that gets efficiently translated and induces UPR [111]. The authors showed that the transcriptional activation of endoplasmic reticulum stress related genes was reduced upon deletion of *Gcn5*, *Ada2* or *Ada3*, whereas deletion of *Ada5* (Spt20) completely abrogated the transcriptional activation [114]. The same group further demonstrated that Ada5 is essential for Ire1 dependent processing of HacI mRNA in vivo, thus proving an important role of ADA complex components in the UPR in yeast [115]. Similar to yeast, it was shown that mammalian SAGA complex plays an important role in endoplasmic reticulum stress related genes [116]. The authors demonstrated that mammalian Spt20, like its yeast counterpart, was indeed a subunit of the SAGA complex [116]. By chromatin immunoprecipitation studies, the authors showed that Spt20 and other SAGA complex components are recruited onto the endoplasmic reticulum stress response genes and knockdown of Spt20 abrogates the endoplasmic reticulum stress response [116]. Again, similar to yeast, the recruitment of Spt20 was shown to be specific to endoplasmic reticulum response genes but not to other stresses [116]. Furthermore, in a recent study it was shown by multidimensional protein identification technology (MudPIT), that mammalian ATF6 transcription factor recruits the SAGA and ATAC complexes onto the endoplasmic reticulum stress response enhancer elements present on endoplasmic reticulum stress response genes and thus are involved in controlling the transcription of these genes [117]. Taken together, these studies point towards a potential role of the ADA complex and its components in UPR.

Further, the STAGA HAT complex has been shown to interact with spliceosome-associated protein 130 (SAP130), a component of the SF3b splicing factor [76].

SF3b gets recruited to pre-spliceosomal complexes in association with U2 snRNP. This points to an important role of STAGA complex in mRNA splicing [76]; however, further studies are required to provide more insights into this function. Another phenomenal study in yeast revealed the role of the SAGA complex and its components Ada2 and Sus1 in tethering of transcriptionally active genes to the nuclear envelope [118]. It has been known that certain genes come closer to the nuclear periphery upon their transcriptional activation. In this study, the authors demonstrated that yeast SAGA complex components Ada2 and Sus1 are involved in confinement of active *GAL* reporter genes to the nuclear periphery [118]. Ada2 and Sus1 achieve this by physically linking the active *GAL* genes to the nuclear pore complex component, Nup1 [118]. Accordingly, deletion of *Ada2* or *Nup1* was shown to abrogate this confinement of *GAL* genes to nuclear periphery [118] suggesting an important role of the SAGA complex in regulating transcription of genes by a novel mechanism of nuclear periphery tethering.

Recent evidence demonstrates mammalian Gcn5 and SAGA complex to be involved in telomere maintenance by controlling the ubiquitination of a component of the shelterin protein complex [119]. Shelterin is a multi-subunit protein complex involved in structural maintenance of telomeres [120]. The authors show that *Gcn5* deletion leads to telomere dysfunction in mammalian cells [119]. This phenomenon was shown to be dependent upon the deubiquitination module of the SAGA complex [119]. The authors demonstrated that the SAGA complex component, ubiquitin-specific protease 22 (Usp22), is involved in deubiquitinating TRF1 (a shelterin complex component) and thus plays an important role in controlling the stability of TRF1 [119]. Interestingly, Gcn5 was shown to be required for association of the Usp22 deubiquitinating module to the SAGA complex and is thus, involved in the maintenance of proper telomere structure through TRF1 [119]. These studies demonstrate the role of the ADA complex components in various important cellular processes and suggest further studies must be carried out to gain more insights into role of the ADA complex components in maintaining genomic integrity.

2.9 The ADA Complex and HAT Inhibitors

As discussed above, acetylation of histones and other proteins plays an important role in a variety of physiological processes in cells, and deregulation of the proteins that regulate acetylation leads to oncogenesis. Consistent with this idea, various laboratories have focused on discovering new synthetic or natural drugs that inhibit enzymes such as HDACs and HATs, which are involved in maintaining homeostasis in acetylation. Inhibitors targeting HDACs have been studied extensively [40, 121, 122]. Many of those inhibitors have shown promising anti-cancer activities without affecting non-cancerous cells, and, accordingly, these inhibitors are currently involved in ongoing clinical trials [40, 121, 122]. Recently, two HDAC inhibitors (HDACi), Vorinostat and Depsipeptide, were approved by the FDA for use as

nti-cancer agents after their validation in cancer patients [122]. Vorinostat was the first HDAC inhibitor to be approved by FDA for the treatment of cutaneous T-cell lymphoma. Many other HDAC inhibitors are in clinical trials for use as anti-cancer drugs, either alone or in combination with other drugs [122]. On the other hand, inhibitors of HATs have seen a slow development, and only in recent years have considerable efforts been made to identify various HAT inhibitors (HATi). The HATi identified till now can be classified into the following three categories: (1) bi-substrate inhibitors, (2) small molecule synthetic inhibitors, and (3) natural compounds [123]. Bi-substrate-based inhibitors include the spermidinyl-CoA-based HAT inhibitors. These inhibitors induce a transient block in DNA replication and impair DNA repair in cancer cells but not in normal cells [124]. However, these compounds alone have been found to be incapable of affecting cancer cell proliferation [124]. Interestingly, these inhibitors were shown to provide cancer-specific chemo- and radio-sensitization due to their ability to affect the DNA repair process [124].

Various natural compounds have been shown to have HAT inhibitory properties. These include anacardic acid (potent inhibitor of p300 and PCAF HAT activity), garcinol (also inhibits HAT activity of both p300 and PCAF), Epigallo Cathenin (present in green tea) and curcumin (a specific inhibitor of p300/CBP HAT activity). Out of these inhibitors, curcumin has been extensively studied for its anti-cancer activities, and various clinical trials involving curcumin are in progress [123]. Furthermore, several small molecule synthetic inhibitors have been designed to inhibit HAT activity such as γ-butyrolactone MB-3 (a GCN5-specific inhibitor), isothiazolones (p300 and PCAF-specific inhibitor) as well as various quinoline derivatives.

Recently, an isothiazole inhibitor NU9056 (Tip60 inhibitor) was shown to have anti-cancer effects on prostate cancer cells [125]. Notably, several of these small molecule inhibitors were designed as analogs of naturally occurring HATi including anacardic acid and garcinol [123]. Although much progress has been made in discovering novel HATi, our knowledge of anti-cancer activities of HATi is limited in comparison with HDACi. This could be attributed to the fact that HATi are less efficient than HDACi, and also because the current HATi doses are not physiologically feasible. Clearly, other than curcumin, which is in clinical trials, HATi need to be improved, and further studies are required to accept these as anti-cancer agents.

2.10 Conclusions and Future Perspectives

Studies carried out at the end of the twentieth century and in the beginning of the twenty-first century have shown the importance of the ADA complex and its components in several important cellular processes in organisms ranging from yeast to humans (Fig. 2.2), thus indicating an indispensible role of these components during evolution. In this chapter we focused on the ADA complex and its components

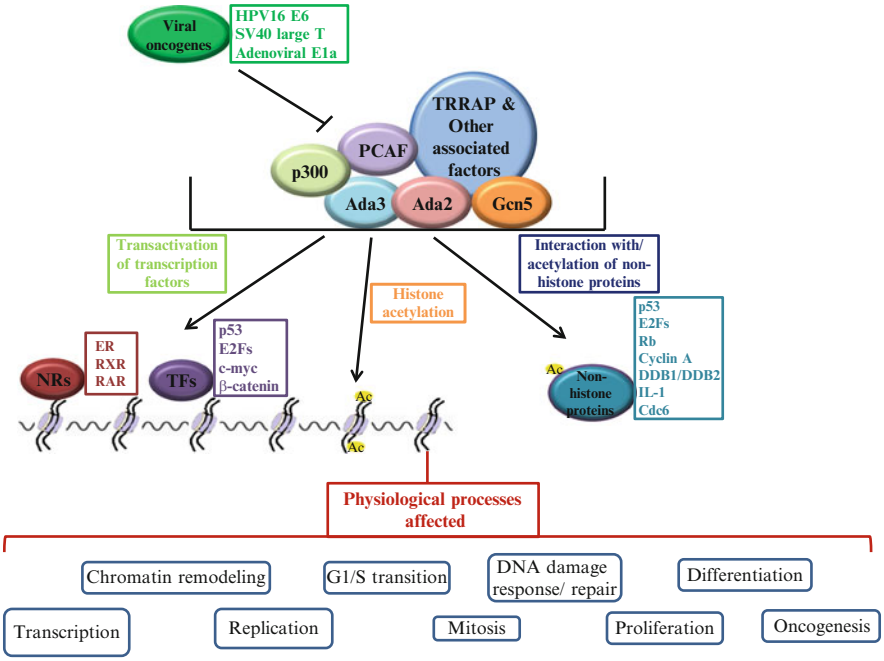

Fig. 2.2 The ADA complex and its components regulate several cellular processes by associating with and/or acetylating various transcription factors (*TFs*), nuclear receptors (*NRs*), histones and non-histone proteins. Viral oncogenes, by associating with different components of ADA complex, disrupt its function

Ada2 and Ada3, which do not seem to have intrinsic HAT activity. We also discussed various important HATs that are present in ADA complex. Although, a distinct functional ADA complex has been shown to be present in yeast, there is no in vivo evidence of such a complex in mammals, clearly indicating that more work is required in this context. Importantly, most known functions of the proteins Ada2 and Ada3 are dependent on their association with HATs. However, it remains possible that these proteins could have HAT-independent functions. Based on the role of these complex components in various important processes, including cancer, several laboratories are now focusing on making and testing new HAT inhibitors (HATi) that could prove useful in treating cancer as well as other diseases. However, novel HATi need to be designed to be specific, to have lower IC50, and to be potent against tumor cells sparing normal cells. Importantly, development of chemical inhibitors that can prevent protein-protein interaction of Ada proteins with HATs is expected to be an alternative strategy to treat cancer. Taken together, although we have made tremendous progress in understanding role of mammalian ADA complex in various physiological processes, more studies particularly in animal models need to be carried out to understand the role of each component in vivo.

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Chapter 3
RUNX2 Transcriptional Regulation in Development and Disease

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Abstract RUNX2, a member of the Runt family of transcription factors, plays important roles in embryonic development to promote osteogenesis and angiogenesis. RUNX2 has been implicated in the promotion of disease, including cleidocranial dysplasia, in cancer progression, and in metastasis of breast and prostate tumors. Its aberrant expression in disease states may be the result of several mechanisms such as haploinsufficiency, mutation, or amplification. In osteogenesis and cancer progression, interactions with core-binding factor-β (Cbf-β) and other cofactors are responsible for the regulation of target gene expression including, but not limited to, VEGF, osteopontin, osteocalcin, MMPs, and BMPs. RUNX2 transcriptional function within cells is regulated by signal transduction events leading to activation of ERK, Smads, cdks, and Akt, which result in phosphorylation, DNA binding, and transcriptional activation or repression of target genes. Constitutive activation of signaling pathways in tumor cells results in aberrant expression and activation of RUNX2. Specific RUNX2 targeting agents, therefore, may bypass the effects of redundant signal transduction pathways within cancer cells and be an effective therapeutic strategy for treatment of RUNX2-positive cancer patients.

Keywords Runx2 • Osteoblast • Cancer • Metastasis • Transcription • Cell cycle • TGF-β • Vitamin D3

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Abbreviations

1,25-OH D3	1,25-OH Vitamin D3
AA	Amino acid
Akt	v-akt murine thymomaviral oncogene homolog 1 (aka: protein kinase B or PKB)
AML	Acute myeloid leukemia
AR	Aldose reductase (Context dependent. Not to be confused with androgen receptor)
AR	Androgen receptor (Context dependent. Not to be confused with aldose reductase)
Atf6	Activating transcription factor 6
BMP	Bone morphogenetic protein
BMP-2	Bone morphogenetic protein-2
BR-DIM	3,3′-diindolylmethane
BSP	Bone sialoprotein (aka: OPN)
C/EBPβ	CCAAT/enhancer binding protein beta
CBFα	Core-binding factor alpha
Cbf-β	Core-binding factor subunit beta
Cbfβ-SMMHC	Core binding factor β – smooth muscle myosin heavy chain
CBP	CREB-binding protein
CCD	Cleidocranial dysplasia
Cdk	Cyclin-dependent kinase
CML	Chronic myeloid leukemia
c-myc	v-myc avian myelocytomatosis viral oncogene homolog
CoAA	Co-activator activator
CSC	Cancer stem cell
CSF2	Colony stimulating factor 2
CST7	Cystatin-7 (aka: cystatin-F or leukocystatin)
CTGF	Connective tissue growth factor
Cx43	Connexin 43
D-ELISA	Deoxyribonucleic acid binding-enzyme-linked immunosorbent assay
DNA	Deoxyribonucleic acid
EC	Endothelial cell
EMT	Epithelial mesenchymal transition
ERK	Extracellular signal-regulated kinase
ERα	Estrogen receptor alpha
EWS-FLI	Ewing's sarcoma breakpoint region 1 t(1122)(q24q12)
FGF2	Fibroblast growth factor 2 (basic)
FGF2R	Fibroblast growth factor 2 receptor
FoxO1	Forkhead box O1
G9a	Histone-lysine N-methyltransferase H3 lysine 9 specific 3 (aka: euchromatic histone-lysine N-methyltransferase)

GPCR	G-protein coupled receptor
HBME	Human bone marrow endothelial cell
HDAC	Histone deacetylase
HG	Hyperglycemia
HIF1-α	Hypoxia inducible factor 1 alpha subunit
HUVEC	Human umbilical vein endothelial cell
IGF-1	Insulin-like growth factor-1
IGF-1R	Insulin-like growth factor 1 receptor
Ihh	Indian hedgehog
IL8	Interleukin 8
JNK	c-jun-N-terminal kinase
MAPK	Mitogen-activated protein kinase
MET	Mesenchymal epithelial transition
miRNA	Micro-ribonucleic acid
MLK3	MAP3K mixed-lineage kinase 3
MM	Multiple myeloma
MMP	Matrix metalloproteinase
MORF	Monocyte leukemia zinc finger related factor
MOZ	Monocyte leukemia zinc finger protein
mRNA	Messenger ribonucleic acid
mSin3a	Mammalian transcriptional corepressors Sin3a
NBIF	Neobavaisoflavone
NLS	Nuclear localization signal
NMTS	Nuclear matrix targeting signal
OPN	Osteopontin (aka: BSP)
$p21^{CIP1}$	Cyclin-dependent kinase inhibitor 1A
p27	Cyclin-dependent kinase inhibitor 1B
p300	E1A binding protein p300
p38	p38 mitogen activated protein kinase
PEBP2α	Polyomavirus enhancer-binding protein 2 alpha
PI3K	Phosphoinositide-3-kinase
PIP	Prolactin-induced protein
PKC	Protein kinase C
PlxnA2	Plexin A2
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homolog
PTHrP	Parathyroid hormone-related protein
RANKL	Receptor activator of nuclear factor kappa-B ligand
RNA	Ribonucleic acid
Rorβ	Retinoid-related orphan receptor beta
rRNA	Ribosomal ribonucleic acid
RUNX1/2/3	Runt-related transcription factor 1/2/3
SDC2	Syndecan 2
SDF1	Stromal cell-derived factor 1
SH3PXD2A	SH3 and PX domain-containing protein 2A

shRNA	Small hairpin ribonucleic acid
siRNA	Small interfering ribonucleic acid
Smad	Sma and mad related proteins
Smurf	Smad specific E3 ubiquitin protein ligase
SNAI2	Snail homolog 2/slug
SNP	Single nucleotide polymorphism
Sox9	Sex determining region Y-box 9 transcription factor
Suv39h1	Suppressor of variegation 3–9 homolog 1
TAZ	Transcriptional coactivator with a PDZ-binding motif
TGF-β	Transforming growth factor beta
TLE-1	Transducin-like enhancer protein 1
TLE2/3	Transducin-like enhancer protein 2/3
UV	Ultraviolet
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor
Wip1	Protein phosphatase magnesium-dependent 1 delta
WWOX	WW domain containing oxidoreductase
WWP1	WW domaincontaining E3 ubiquitin protein ligase 1
YAP	Yes-associated protein

3.1 Introduction

The *RUNX* genes are a family of transcription factors originally identified in *Drosophila* [1, 2]. There are three mammalian *RUNX* genes encoding the proteins RUNX1, RUNX2, and RUNX3. Historically, the literature contains many different nomenclatures for the mammalian RUNX family proteins. RUNX2(Runt related transcription factor 2) is also AML-3 (acute myeloid leukemia-3), PEBP2α (polyoma enhancer binding protein 2α), and CBFα (core binding factor α) [2–5]. The *RUNX* genes control many normal cellular processes including hematopoiesis (*RUNX1*), osteogenesis (*RUNX2*), and epithelial and neuronal development (*RUNX3*) [1]. *RUNX* genes encode several evolutionarily conserved proteins. There are four *RUNX* genes in zebra fish, four in *drosophila*, one in sea urchins, and one in *C. elegans* to name a few [2]. High conservation of proteins through evolutionary history generally suggests an extremely essential biological function.

The RUNX proteins are members of a heterodimeric complex composed of an α and β subunit. The *runt* domain genes contain the DNA binding α subunit [1]. The β subunit, consisting of core-binding factor-β (Cbf-β), binds to the runt domain within RUNX proteins (Fig. 3.1) to help stabilize RUNX-DNA interactions [1]. In addition, Cbf-β protects RUNX proteins from phosphorylation and degradation via the proteasome [1]. The RUNX proteins share many common protein domains (Fig. 3.1). The runt domain, which is responsible for binding DNA, is located in the N-terminus of the protein and is composed of 128 amino acids [3]. The runt domain is the most highly conserved domain among members of the RUNX family including the *Drosophila* ortholog *runt*. Located C-terminal to the runt domain is the nuclear

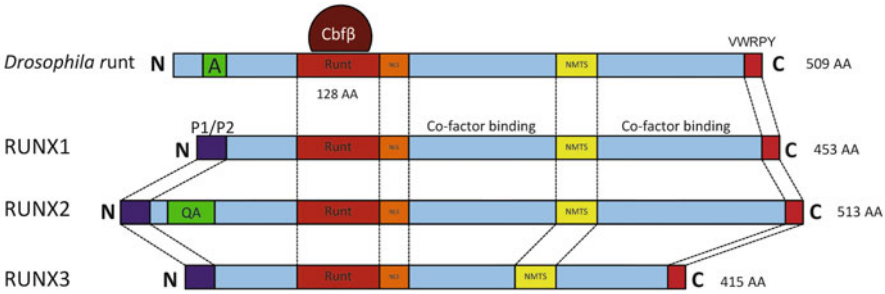

Fig. 3.1 Mammalian RUNX isoforms protein structure. The three mammalian RUNX proteins share domains with *drosophila runt*. N terminal (*purple*) are the P1 and P2 promoters. The Runt domain (*red*) is the DNA binding and Cbf-β interaction domain. C-terminal of the Runt domain (*orange*) is the nuclear localization sequence (NLS) controlling nuclear translocation of RUNX proteins. C-terminus (*light blue*) are a variety of sequences mediating co-factor binding to either activate or repress RUNX transcription of target genes. The nuclear matrix target sequence (NMTS; *yellow*) controls sub-nuclear localization of RUNX proteins

matrix targeting signal (NMTS) which is responsible for sub nuclear localization of RUNX proteins [1, 6]. This domain is comprised of 38 amino acids [5] folding into a loop-turn-loop tertiary structure [6]. Mutations within this domain reveal the essential nature of its function: without a functional NMTS, RUNX proteins cannot be transactivated or localized in foci within the nucleus [5]. Mutations within the second loop of the NMTS have been shown to inhibit RUNX2 interaction with the nuclear matrix [6] leading to compromised gene regulation. The nuclear localization signal/sequence (NLS) is comprised of 9 amino acids [5] and maintains RUNX localization to the nucleus. Furthermore, the C-terminus of RUNX proteins contains binding sites for corepressors and coactivators [1], which modulate RUNX activity. Depending on the cell stimulus or the cell type, different modulators of transcription are able to bind the RUNX proteins and either enhance or repress transcription.

All of the RUNX proteins have been implicated in disease (see Fig. 3.2 for diseases in which RUNX2 has been shown to play a role). Their normal functions are altered via mutations, epigenetic silencing, chromosomal translocation, cellular mislocalization, or by gene amplification. There is evidence that the RUNX proteins function as both tumor suppressors and as oncogenes depending on the disease context.

Although RUNX1 is not the largest protein isoform in mammals, it does encompass the largest genomic coding and regulatory region of 260 kb of DNA, comprised of 11 exons [4] encoding for 453 amino acids [1]. Within cells, RUNX1 functions to maintain normal hematopoiesis [1]. It is the target of numerous mutations and chromosomal translocations in hematological malignancies such as leukemia. In most reported cases of leukemia (specifically acute myeloid leukemia) where RUNX1 translocations are discovered, there appears to be a dominant negative function resulting from the new RUNX1-fusion protein. RUNX1 translocations and mutations are seen in acute myeloid leukemia (AML), blast crisis of chronic myeloid leukemia (CML), and acute lymphoblastic leukemia (ALL) [4]. There have been a few reports of gain of function mutations in RUNX1 as a result of an extra copy of

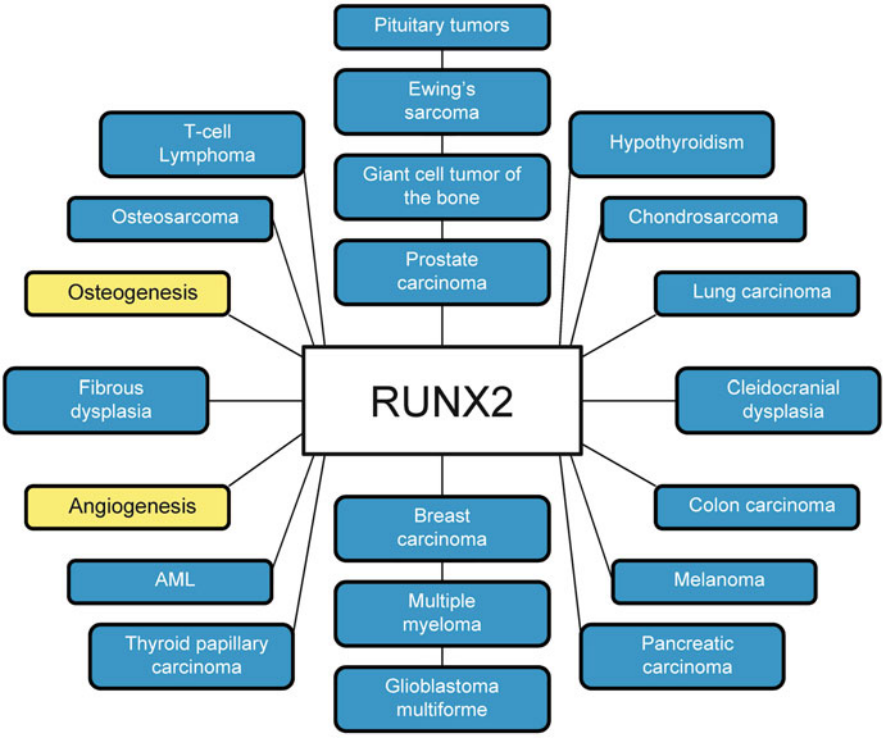

Fig. 3.2 RUNX2 in development and disease. Normal functions of RUNX2 (*yellow*) and diseases in which RUNX2 has been shown to play a role (*blue*)

the RUNX1 gene. This has been reported in Down Syndrome-related acute megakaryoblastic leukemia [4]. Knockout mice display a wide range of defects including, but not limited to, megakaryocyte defects, T-cell defects, myeloproliferative diseases, as well as T-cell lymphomas [2]. New studies have suggested a role for RUNX1 in endochondral ossification to mediate fracture healing in bone [7].

The smallest of the three mammalian RUNX proteins, RUNX3, is also thought to be the most primitive in evolutionary history, spanning 67 kb of DNA and composed of six exons [4] translating to 415 amino acids [1]. Expressed ubiquitously throughout the body, it can be found within the epithelia, mesenchyma, blood cells, dorsal root ganglion neurons, and predominantly in the gut epithelia [4]. RUNX3 has been shown to be essential for proper gut epithelial and neuronal development [8]. RUNX3 is essential for proprioceptive neuron axon path finding in the spinal cord [2] and there have also been reports of RUNX3 regulating CD4 silencing in T-cells [2]. Within the gut epithelia, research demonstrates that inactivation of RUNX3 leads to hyperplasia with a loss of response to transforming growth factor-β (TGF-β) inhibition [4] resulting in gastric cancers [8]. Inactivation has been described to occur through mutation, epigenetic silencing, hemizygous deletion, or cytoplasmic mislocalization.

Human RUNX2, located on the short arm of chromosome 6 at position 21 (6p21) [3], is the largest family member containing 513 amino acids [1] and has unique domains not present in the other mammalian isoforms (RUNX1 and RUNX3); one in the N-terminus and one in the C-terminus (see Fig. 3.1). RUNX2 is expressed early in embryonic development in mesenchymal stem cells [5]. During mouse embryogenesis, RUNX2 mRNA has been detected as early as E11.5 in the limb buds and the condensation of the humerus [5]. There is very weak expression observed as early as E9.5 in the notochord [5]. RUNX2 is the master regulator of osteoblast differentiation and chondrocyte maturation in a process called osteogenesis [1, 5]. RUNX2 controls the commitment of mesenchymal stem cells to the osteoblast lineage and has been shown to be abnormally expressed in adult tissues, leading to disease. Haploinsufficiency of RUNX2 promotes cleidocranial dysplasia (CCD) [9, 10]. In addition, there have been a few reports of RUNX2 mutations occurring within the runt domain which also result in CCD [5]. The oncogenic potential of RUNX2 was first identified from its ability to synergize with c-myc in T-cell lymphoma development [8, 127]. Aberrantly expressed RUNX2, normally at non-detectable to low levels in epithelial tissue, is thought to promote bone metastasis through activation of genes in malignancies such as breast and prostate cancer [8]. These target genes include, but are not limited to, vascular endothelial growth factor (VEGF), osteopontin (OPN), osteocalcin (OC), and matrix metalloproteinases (MMP's) [8]. The rest of this review will focus on RUNX2.

3.2 RUNX2: A Master Transcription Factor

3.2.1 Function in Osteogenesis and Angiogenesis

Osteogenesis consists of intramembranous ossification (bone) and endochondral ossification (cartilage) [5]. Bone homeostasis is an important process that requires a balance between bone formation (osteoblasts) and resorption (osteoclasts). Osteoblasts are responsible for laying down new bone matrix in addition to the mineralization of the new bone matrix [11]. Osteoblasts also stimulate the differentiation of osteoclasts while osteoclasts produce factors which digest the mineralized bone matrix [11]. RUNX2 is the master regulator of osteoblast differentiation and osteogenesis [132]. RUNX2 expression is controlled by two promoters: P1 and P2 early in osteoblast differentiation [12]. As differentiation progresses RUNX2 protein levels do not increase, but rather, the transcriptional activity level increases [11]. Experiments in knockout mice show the essential role of RUNX2 in bone formation: knockout mice die soon after birth because of asphyxiation as a consequence of a lack of skeletal formation [5, 11]. In addition, analyses reveal that these mice lack mature osteoblasts thereby inhibiting the formation of any bone matrix or osteoclast differentiation.

Since osteoblasts lay down bone matrix to form mineralized bone and osteoclasts break down the matrix to resorb bone; RUNX2 indirectly controls osteoclast

differentiation. Receptor activator of nuclear factor kappa-b ligand (RANKL) promotes osteoclast maturation and is also a RUNX2 target gene [5]. Cells lacking RUNX2 express less RANKL and, therefore, there is less osteoclast maturation [5] and less bone resorption. Research has further shown that while endochondral ossification is delayed in RUNX2 knockout mice, it does eventually occur [5, 130, 134]. Therefore, there is redundancy and other factors are able to compensate for the lack of RUNX2 during endochondral ossification.

The RUNX2 transcription factor is also a regulator of angiogenesis in bone development [13, 14], is expressed in vascularizing adult tissues [15], and promotes tumor metastasis [16, 17]. It interacts with its heterodimeric partner, Cbf-β, and with hypoxia-inducible factor 1-α (Hif1α) to activate the major angiogenic factor, VEGF [18]. RUNX2 is a transcriptional activator of specific target genes that promote angiogenesis, such as MMPs [19]. Conversely, it represses the cell cycle inhibitor $p21^{Cip1}$ and increases endothelial cell (EC) or cancer cell proliferation [20, 21]. Our laboratory has found that glucose metabolism, autocrine IGF-1 signaling, and phosphorylation by cyclin-dependent kinases, regulate RUNX2 DNA-binding activity, angiogenic target genes, EC proliferation, tube formation, and wound healing [20, 22–25, 138]. However, exposure of EC to hyperglycemia (HG) activated the aldose reductase (AR) polyol pathway, which increased oxidative stress and inhibited RUNX2 DNA binding [22].

3.2.2 RUNX2 in Disease

Although RUNX2 regulates osteogenesis and angiogenesis [1, 12], aberrant expression of RUNX2 can lead to disease (Fig. 3.2). Cleidocranial dysplasia (CCD) is a disease in which there are abnormalities in bone and dental development [5, 9, 26, 27]. Haploinsufficiency [1, 9] of RUNX2 has been shown to be a leading cause of CCD. In addition, there are mutations in RUNX2 that lead to CCD [26]. A heterozygous single-base deletion resulting in a premature stop codon in the runt domain produces a truncated form of RUNX2 [26] that is unable to bind DNA and control transcription of essential target genes. This mutation was not found in normal individuals or non-CCD subjects.

RUNX2 has been proposed as a biomarker in numerous cancers [1–4, 16, 17, 28, 29, 36–38, 41, 43, 45–49, 53, 54, 61, 94, 127–129, 131, 135] to evaluate the promotion of cancer cell metastasis to the bone. Sase et al. showed that RUNX2 expression was significantly associated with human colon carcinoma progression [28, 29]. In colorectal cancer RUNX2 is not only amplified, but the *RUNX2* gene also contains genetic variations termed single nucleotide polymorphisms or SNPs. In cases of colorectal carcinoma, Slattery et al. found a total of 19 SNPs in *RUNX2* [29].

One tumor in which both mRNA and protein levels of RUNX2 have been shown to be elevated is osteosarcoma [24, 30–35]. Osteosarcoma is a very aggressive pediatric cancer of the bone with a highly heterogeneous phenotype [32, 33, 35]. In osteosarcoma and chondrosarcomas, RUNX2 expression was found to positively correlate with bone morphogenetic protein-2 (BMP-2) mRNA levels [27]. Cell cycle

deregulation of RUNX2 led to osteosarcoma pathogenesis [24]. High levels of RUNX2 in osteosarcoma resulted in high rates of metastasis and a poor survival rate, supporting the notion of RUNX2 as a good prognostic marker [35]. RUNX2 was also the only upregulated marker in osteosarcoma that exhibited a positive correlation with chemotherapeutic resistance [33].

RUNX2 expression correlates with unfavorable prognoses in prostate cancer [36] and has been found to be upregulated in both breast and prostate cancers [8, 16, 25, 37–46, 128, 129]. In breast and prostate cancer cell lines RUNX2 was shown to enhance cell motility [41]. Unfortunately, many of the RUNX2 functions are cell type specific making it difficult to discern a universal function in disease progression. In MDA-MB-231 breast cancer cells, knockdown of RUNX2 had no effect on cell growth and proliferation whereas in MCF7 breast cancer cells RUNX2 enhanced cell proliferation upon growth factor deprivation [41]. In MCF-10A cells RUNX2 disrupts normal mammary acini formation in suspension culture [47]. Using electron microscopy, an absence of lumen formation was noted, possibly due to an increase in cell proliferation, decreased apoptosis, and a loss of basement membrane formation which is dependent on RUNX2 expression [47]. These phenotypes can be reversed in MDA-MB-231 cells using siRNA to deplete RUNX2 [47]. The consensus of data supports an oncogenic function for RUNX2 in breast cancer. However, there is a report suggesting RUNX2 functions as a tumor suppressor in breast cancer [48]. There have been a few reports implicating RUNX2 in promoting the formation of hematological malignancies [131] such as myeloid leukemia [49]. For example, RUNX2 cooperates with the fusion protein, Cbf-β-SMMHC, to promote leukemia development [49] and haploinsufficiency of RUNX2 delays the onset of acute myeloid leukemia [49].

3.3 Transcriptional Regulation: Target Genes and Cofactors

3.3.1 RUNX2 Target Genes

As a DNA-binding factor RUNX2 controls the transcription (through activation and repression) of numerous genes important in normal tissue homeostasis (Table 3.1). Many of these genes are abnormally activated in disease states, including cancers, and enable cancer cells to survive and metastasize to distant niches.

In osteoblasts, RUNX2 activates expression of the essential osteogenesis signaling factor: bone morphogenetic protein-2 (BMP-2) [50]. BMP-2 is a member of the transforming growth factor-β (TGF-β) superfamily of signaling molecules. It binds to TGF-β receptors, leading to activation of intracellular signaling via Smad-dependent and/or Smad-independent pathways ultimately resulting in further RUNX2 activation and promotion of a feed-forward loop. In MC3T3-E1 cells, BMP-2 was shown to enhance RUNX2 association with the promoter of Atf6 [51]. Atf6 is another transcription factor which is known to mediate osteoblast differentiation in a RUNX2 dependent manner [51]. Specifically, BMP-2-stimulated

Table 3.1 Runx2 regulated genes

Gene name	Gene symbol	Basic function	References
Bone morphogenetic protein-2	BMP-2	TGF-beta superfamily protein; cell signaling events; important for development of bone and cartilage	[50]
Snail1	SNAI1	Zinc finger transcription factor; promote and EMT through repression of E-Cadherin transcription	[99]
p27 (cyclin-dependent kinase inhibitor 1B)	CDKN1B	Controls G1 arrest; degradation is required to transition the cell from quiescence to proliferation	[80]
Bone sialoprotein 1 (osteopontin)	BSP1 (OPN)	Extracellular matrix component of the bone	[45, 59, 100]
Ribosomal ribonucleic acid	rRNA	RNA component of the ribosome; functions in protein synthesis	[57]
Matrix metalloproteinase 9 (gelatinase B)	MMP9	Functions in extracellular matrix degradation	[36–38, 44, 100, 101]
Stromal cell-derived factor 1	SDF1/CXCL12	Cytokine/chemokine signaling factor functioning in migration and adhesion	[36, 38, 44]
Cystatin F	CST7	Protease inhibitor; functions in immune regulation; promotes tumor metastasis	[38, 44]
Matrix metalloproteinase 13	MMP13	Functions in extracellular matrix degradation	[36, 37, 44, 52–55]
Prolactin-induced protein	PIP	Water transport; binds immunoglobulins; used as a biomarker in breast cancer; promotes cellular proliferation	[39, 44]
Vascular endothelial growth factor	VEGF	Promotes angiogenesis	[36, 37, 102, 103]
Insulin-like growth factor-binding protein 5	IGFBP5	Prolong the half-life of IGF's and alters IGF interactions with IGFR to promote cell survival, growth, and proliferation	[104]
Collagen type X alpha 1	Col10a1	Extracellular matrix component of the bone	[105]
Transcription factor Sox9	Sox9	Regulates transcription of genes important in osteogenesis	[36, 38]
Lipocalin-2	LCN2	Important for innate immunity; upregulated during inflammation; functions in apoptosis	[36]
Snail2	SNAI2	Zinc finger transcription factor; promotes oncogenic transformation of epithelial cells	[36, 38, 106]
Pepsinogen C (gastricsin)	PGC	Extracellular matrix degradation and invasiveness	[36]
Endothelin 2	EDN2	Important for angiogenesis	[36]
Receptor activator of nuclear factor kappa-b ligand	RANKL	Also called osteoclast differentiation factor; receptor that serves to activate osteoclasts; aids in metastasis to the bone	[36]

Parathyroid hormone-related protein	PTHrP	Regulator of cell growth, differentiation, migration, and survival; aids in bone metastasis	[36, 37, 64]
Interleukin 8	IL8	Cytokine/chemokine signaling; Potent activator of angiogenesis	[36, 37]
Sphingosine kinase 1	SPHK1	Regulates proliferation and survival; aids in metastasis to the bone	[36, 38]
Endothelial differentiation GPCR 3	EDG3	Receptor for sphingosine 1-phosphate; aids in metastasis to the bone	[36]
Kallikrein-2	KLK2	Cleaves PSA to active form; prognostic marker for prostate cancer risk	[36]
Ras small GTPase, Rab type	RAB3B	Modify membrane lipids and small GTPases of the Rab family; secretory pathway	[36]
Ras small GTPase, Rab type	RAB43	Modify membrane lipids and small GTPases of the Rab family; secretory pathway	[36]
Ras small GTPase, Rab type	RAB35	Modify membrane lipids and small GTPases of the Rab family; secretory pathway	[36]
Activating transcription factor 6	ATF6	Involved in the unfolded protein response; activates the transcription of ER chaperones	[51]
Bcl-2-associated X protein	Bax	Promotes apoptosis	[30, 107]
Exostosin-1	Ext1	Tumor suppressor; glycosyltransferase	[72]
Heparanase	HPSE	Helps degrade heparan sulfate; promotes tumor angiogenesis and metastasis	[72]
Bone morphogenetic protein - 3b	BMP-3B	Member of the TGF-beta superfamily of secreted molecules; aid in metastasis to the bone	[108]
Thyroglobulin	Tg	Used by thyroid to make T4 and T3; can be used as a biomarker in thyroid cancers	[109]
Cytochrome P450 11a1	CYP11a1	Converts cholesterol to pregnenolone; aids in cancer progression; sterol/steroid metabolism	[110]
Cytochrome P450 39a1	CYP39a1	Converts cholesterol to bile acids; aids in cancer progression; sterol/steroid metabolism	[110]
Cytochrome P450 51	CYP51	Converts lanosterol to cholesterol; aids in cancer progression; sterol/steroid metabolism	[110]
Lanosterol synthase	Lss	Functions in sterol/steroid metabolism	[110]
7-dehydrocholesterol reductase	Dhcr7	Functions in sterol/steroid metabolism	[110]
MicroRNA cluster	miR23a-27a-24-2	MicroRNA cluster that promotes apoptosis; downregulated during tumor progression	[85]
Regulator of G-protein signaling 2	Rgs2	Functions in signaling; drug resistance; tumor angiogenesis	[111]
Regulator of G-protein signaling 4	Rgs4	Functions in signaling; drug resistance; tumor angiogenesis	[111]
Regulator of G-protein signaling 5	Rgs5	Functions in signaling; drug resistance; tumor angiogenesis	[111]
Regulator of G-protein signaling 16	Rgs16	Functions in signaling; drug resistance; tumor angiogenesis	[111]

(continued)

Table 3.1 (continued)

Gene name	Gene symbol	Basic function	References
G-protein coupled receptor 23	Gpr23	Function in G-protein coupled signaling to activate cAMP signaling and PI3K to promote cell survival, growth, and proliferation	[111]
G-protein coupled receptor 30	Gpr30	Function in G-protein coupled signaling to activate cAMP signaling and PI3K to promote cell survival, growth, and proliferation	[111]
G-protein coupled receptor 54	Gpr54	Function in G-protein coupled signaling to activate cAMP signaling and PI3K to promote cell survival, growth, and proliferation	[111]
G-protein coupled receptor 64	Gpr64	Function in G-protein coupled signaling to activate cAMP signaling and PI3K to promote cell survival, growth, and proliferation	[111]
G-protein subunit alpha-13	Gna13	Function in G-protein coupled signaling to activate cAMP signaling and PI3K to promote cell survival, growth, and proliferation	[111]
Pannexin3	PANX3	Structural component of gap junctions	[112]
Survivin	BIRC5	Member of the inhibitor of apoptosis family; functions in apoptosis; inhibit caspases	[113]
Galectin-3	LGALS3	Functions in cell adhesion, cell activation and chemoattraction, cell growth and differentiation, cell cycle, apoptosis	[114, 115]
Connective tissue growth factor	CTGF	Stimulates extracellular matrix production, chemotaxis, proliferation and integrin expression; angiogenesis	[56]
Lymphoid enhancer-binding factor 1	Lef1	Participates in Wnt signaling; promotes an EMT by repressing E-Cadherin	[116]
Dual specificity protein phosphatase 1	DUSP1	Negative regulator of cell proliferation	[38]
Osteoprotegerin	OPG	Cytokine receptor; decoy RANKL receptor; regulator of inflammation, innate immunity, and cell survival and differentiation	[114]
Aromatase	CYP19a1	Functions in sterol/steroid metabolism	[100]
Osteocalcin	OC	Extracellular matrix component of the bone	[100, 114, 117, 118]
Type 1 collagen	COL1a2	Extracellular matrix component	[100, 117]
Sclerostin	SOST	BMP antagonist; inhibits Wnt signaling; antagonizes bone formation	[42]
Interleukin 11	IL-11	Functions in cytokine/chemokine signaling; promotes bone cell proliferation and differentiation	[42]
Granulocyte-macrophage colony-stimulating factor	GM-CSF	Cytokine/chemokine signaling; regulates growth and differentiation	[36, 38, 44, 77]

Syndecan 1	SDC1	Integral cell membrane protein to participate in cell migration, proliferation and cell-matrix interactions	[72]
Syndecan 2	SDC2	Integral cell membrane protein to participate in cell migration, proliferation and cell-matrix interactions	[38, 72]
Syndecan 3	SDC3	Integral cell membrane protein to participate in cell migration, proliferation and cell-matrix interactions	[72]
Twinfilin	TWF	Regulations cytoskeletal dynamics via actin	[38]
SH3 and PX domain-containing protein 2A	SH3PXD2A	Involved in extracellular matrix degradation and cellular invasion	[38]
Alkaline phosphatase	ALP	Dephosphorylate proteins	[117]
Glypican	GPC	Modify signaling pathways and contribute to cell proliferation and tissue growth	[72]
Versican	VCAN	Functions in cell adhesion, migration, and proliferation	[72]
Axin2	AXIN2	Functions within the Wnt signaling pathway to maintain Beta-catenin	[119]
Smad 3	SMAD3	EMT and cell transformation	[38]
Dexamethasone-induced ras-related protein 1	RASD1	Plays a role in altered cell morphology, growth and extracellular matrix interactions	[38]
Fibroblast growth factor receptor 2	FGFR2	Receptor tyrosine kinase receptor that regulates cell signaling controlling cellular proliferation and differentiation	[72]
Fibroblast growth factor receptor 3	FGFR3	Receptor tyrosine kinase receptor that regulates cell signaling controlling cellular proliferation and differentiation	[72]
B-cell lymphoma-2	Bcl-2	Protects cells from apoptosis via maintaining mitochondrial membrane integrity; prevents cytochrome c release from the mitochondria	[8]
Prostate specific antigen	PSA	Protease to dissolve coagulum; serine protease; prostate cancer biomarker	[36]
Membrane type 1 matrix metalloproteinase	MT_1MMP	Promotes tumor growth, tumor invasion, and angiogenesis; extracellular matrix protease	[1]
Cyclin D1	CCND1	Regulate CDK kinases during cell cycle; functions as a regulatory subunit for CDK4 and CDK6 during G1/S transition; contributes to tumorigenesis via altered cell cycle	[1]
Cyclin dependent kinase inhibitor 1A	p21	Regulates cell cycle progression at G1 by inhibiting the activity of CDK4 and CDK2	[1]
Cyclin dependent kinase inhibitor 2A	p14	Induce cell cycle arrest at G1 and G2 phases; acts as a tumor suppressor	[1]

RUNX2 activation of Atf6 enables the bone extracellular matrix, osteocalcin, gene to be expressed. A dominant negative construct of Atf6 was shown to inhibit RUNX2 activation of the osteocalcin promoter [51]. However, by restoring wild type Atf6, osteoblasts were able to differentiate and the expression of osteocalcin was restored.

During cancer progression, cancer cells express many bone specific proteins that mediate metastasis to the bone. Some of these proteins are regulated by RUNX2 transcriptional activity and mediate migration/motility and adhesion. Bone sialoprotein (BSP) and osteopontin (OPN) are two factors which mediate breast cancer metastasis to the bone and are activated by RUNX2 in breast cancer cells [45]. Using siRNA technology, Reufsteck et al. were able to demonstrate that targeting BSP and OPN drastically inhibits migration of MDA-MB-231 breast cancer cells when injected into athymic nude mice [45]. In prostate cancer, RUNX2 has been shown to upregulate genes not only associated with increased migration but genes associated with angiogenesis, epithelial-mesenchymal-transition (EMT), membrane trafficking/secretion, and osteolysis [36]. RUNX2 upregulates secretion factors including PTHrP, IL8, CSF2, and SDF-1 (See Table 3.1 for a description of protein function) [37, 38]. RUNX2 also upregulates MMP9, MMP13, VEGF, osteopontin, CST7, Sox9, SNAI2, Smad3, SDC2, Twinfilin, and SH3PXD2A [37, 38]. RUNX2 promoter occupancy in osteosarcoma was examined to determine potential RUNX2 target genes enabling progression of osteosarcoma. In SAOS-2 osteosarcoma cells, knockdown of RUNX2 resulted in an inhibition of motility [34]. RUNX2 upregulated matrix metalloproteinases during cancer progression to promote cell migration from primary tumor sites. MMP13 and MMP9 are two common MMP's upregulated by RUNX2 to mediate cancer cell invasion and metastasis [1, 52–55].

In addition to epithelial cells, RUNX2 has been shown to modulate gene expression in vascular cells. In human aortic smooth muscle cells and C3H10T1/2 cells RUNX2 inhibited the expression of connective tissue growth factor (CTGF) [56]. This is important for endothelial cells since CTGF has been shown to be a contributing factor to the development of atherosclerosis. Therefore, RUNX2 may protect the vasculature from development of atherosclerosis. Knockdown of RUNX2 enhanced CTGF expression [56] in a TGF-β dependent manner. Further analysis of the CTGF promoter revealed Smad binding elements that were able to interact with RUNX2/Smad3 heterodimers upon stimulation of the TGF-β receptors to inhibit transcription [56].

3.3.2 RUNX2 Cofactors

Regulation of gene expression requires a transcriptional complex composed of RNA polymerase, transcription factors, and corepressors or coactivators. RUNX2 C-terminal domain contains many binding sites for both corepressors and coactivators (Table 3.2).

One group of proteins that function as strong corepressors of RUNX2 are histone deacetylases (HDACs). In osseous cells, HDAC1 has been shown to interact with

Table 3.2 Runx2 cofactors

Cofactor	References
CBP	[50]
p300	[99]
HDAC1	[57, 59]
G9a	[44]
HIF1α	[102]
p38	[62]
SMAD	[56, 62, 63, 83, 111]
CBFβ	[25, 42, 120]
Rorβ	[121]
YAP	[21, 122]
TAZ	[91, 123]
C/EBPβ	[52]
Wip1	[107]
WWP1	[87]
ERα	[100, 129]
FOXO1	[61]
AR	[39, 46, 124]
WWOX	[31, 125]
EWS-FLI	[126]
TLE1	[58, 73]
CoAA	[119]
CBFβ-SMMHC	[49]
Gli2	[64]
HDAC7	[5, 60]
Suv39h1	[108]
mSin3a	[122]
TLE2	[122]
TLE3	[122]
HDAC6	[5, 122]
HDAC4	[5, 122]
HDAC3	[5, 122]
Smurf1	[5]
Schnurri-3	[5]
MOZ	[5]
MORF	[5]
HDAC5	[5]

RUNX2 to inhibit ribosomal RNA (rRNA) gene expression [57] thus inhibiting cellular proliferation and protein synthesis. Knockout of HDAC1 was shown to alleviate the RUNX2-mediated repression of rRNA expression resulting in an increase in cell proliferation and overall protein synthesis [57]. Transducin Like Enhancer-1 (TLE-1), functions to also promote RUNX2 inhibition of rRNA gene synthesis during mitosis [58]. In C3H10T1/2 cells HDAC1 bound to RUNX2 inhibited expression of osteopontin, thus inhibiting both proliferation and differentiation of the osteoblast cell [59]. HDAC7 was also shown to be a potent inhibitor of RUNX2 transcriptional activity [60] and HDAC5 was shown to repress RUNX2 expression [5].

In prostate cancer the forkhead box O (FoxO1) protein was found to be a corepressor of RUNX2 [61]. Inhibition of RUNX2 by upregulation of PTEN or FoxO1 protein inhibited prostate cancer cell migration and invasion. In prostate cancer specimens, immunohistochemistry revealed an inverse relationship between RUNX2 and FoxO1 nuclear localization [61]. PTEN inactivating mutations are often seen in prostate cancer [61] and therefore would potentiate RUNX2 activity to promote prostate cancer cell migration and invasion.

While some cofactors function as coactivators and corepressors a few are also able to function as both in a gene-dependent manner. One group used a doxycycline-regulated RUNX2 expression system in C4-2B prostate cancer cells to show that G9a (histone methyltransferase) is able to function as a corepressor for RUNX2 target genes MMP9, CSF2, SDF1, and CST7 [44]. However, G9a functions as a coactivator for RUNX2 transcription of MMP13 and PIP in the C4-2B prostate cancer cell line [44].

3.4 Transcriptional Regulation: Activation and Repression

3.4.1 Regulation of RUNX2 Activity

Several intracellular signaling pathways have been shown to modulate RUNX2 activity (Fig. 3.3). When RUNX2 becomes activated it is able to bind DNA and either promote or inhibit the transcription of its target genes (Refer to Table 3.1). Mitogen-activated protein kinase (MAPK) and TGF-β intracellular signaling are both essential to RUNX2 activation to promote osteoblast differentiation or tumor progression. TGF-β/BMP signaling is a highly important signaling axis used by numerous cells to inhibit cell growth and proliferation under normal conditions. In cancer, tumor cells escape the inhibitory effects of TGF-β resulting in unrestricted proliferation. TGF-β/BMP intracellular signaling can occur via canonical Smad-dependent or non-canonical Smad-independent pathways. Upon stimulation, TGF-β receptors initiate intracellular signaling events leading to Smad activation (canonical TGF-β pathway), nuclear translocation, and Smad interaction with RUNX2 [6, 56, 62–64]. In osteogenesis, TGF-β/BMP signaling is responsible for activating RUNX2 to promote osteoblast differentiation [62, 65]. Conversely, in PC3 prostate cancer cells TGF-β cooperates with RUNX2 to promote cellular growth [46].

A potent regulator of RUNX2 activity is the extracellular signal-regulated kinase, ERK [66, 67, 138, 139]. ERK is a classical MAPK activated upstream in response to numerous extracellular factors including growth factors, cytokines, and G-protein coupled receptor ligands. MAPK signaling is essential for normal bone development. In many cancers where RUNX2 is aberrantly expressed, MAPK signaling is also altered generally through inactivating or activating mutations to components within the signaling cascade. In preosteoblast MC3T3-E1 cells it was demonstrated that S301 and S319 [66], two ERK phosphorylation sites on RUNX2, are

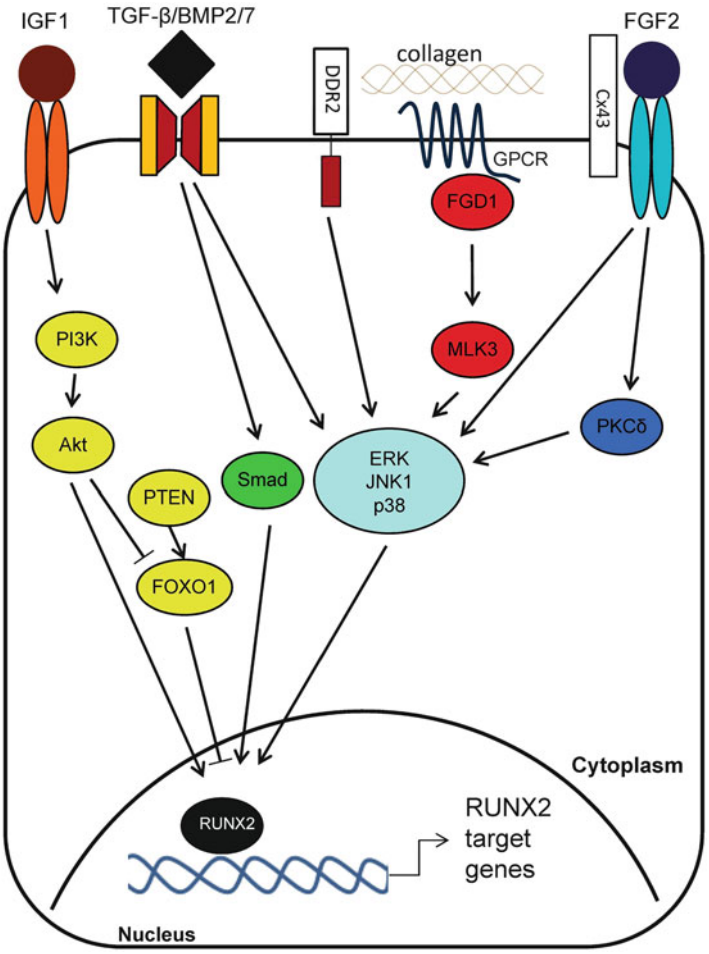

Fig. 3.3 RUNX2 and signal transduction. Many signaling pathways lead to activation or repression of RUNX2 transcriptional activity. These Pathways converge onto the MAPK pathway, the TGF-β/Smad pathway, and the PI3K/Akt pathway

phosphorylated upon nuclear translocation of ERK [67]. MAP3K mixed-lineage kinase 3 (MLK3) has been shown to activate ERK and p38 (another classical MAP kinase). Activation of ERK via MLK3 results in RUNX2 phosphorylation and subsequent osteoblast differentiation [68]. Insulin-like growth factor-1 (IGF-1) binding to IGF-1R (IGF-1 receptor tyrosine kinase) has been shown to be essential for normal skeletal development [69]. IGF-1 regulates the activation of RUNX2 via activation of ERK resulting in phosphorylation of RUNX2 [22, 69, 138]. p38, activated via several extracellular factors, has been implicated in modulating RUNX2 activity levels. p38 activation from TGF-β/BMP signaling (non-canonical TGF-β pathway) has been shown to mediate RUNX2 phosphorylation to promote osteoblast differentiation [62].

Fibroblast growth factor 2 (FGF2) is another extracellular growth factor implicated in promoting osteoblast differentiation through activation of RUNX2 [70–72]. In activating RUNX2, FGF2 functions as a double-edge sword. FGF2 activates protein kinase C (PKC), which in turn increases ERK activity, leading to RUNX2 phosphorylation in MC3T3 osteoblasts [71]. FGF2 also activates ERK through PKC-independent mechanisms (refer to Fig. 3.3). The gap junction protein, connexin-43 (Cx43), enhances this activation via stabilization of FGF2 to FGF2R (refer to Fig. 3.3). An inhibitor of gap junctions, 18β-glycyrrhetinic acid, attenuated the enhancement in RUNX2 transcriptional activity in MC3T3 osteoblasts [71]. In breast cancer MCF7 cells, FGF2 was able to increase BSP expression, which in turn upregulated RUNX2 mRNA [70].

Not all intracellular signaling pathways that lead to RUNX2 phosphorylation result in RUNX2 activation. The c-jun-N-terminal kinase (JNK) is activated by BMP-2 signaling in both C2C12 multipotent cells and MC3T3-E1 preosteoblastic cells [73]. JNK is able to phosphorylate RUNX2 at S104 [73] resulting in inhibition of RUNX2 activity and thus preventing osteoblast differentiation. Inhibition of JNK via a dominant negative JNK1, JNK knockdown, or treatment with a JNK inhibitor counteracted this inhibition to enable osteoblast differentiation.

RUNX2 has also been shown to play a role in endothelial signaling and cell cycle progression in response to physiological levels of glucose [22]. Euglycemic conditions were able to restore RUNX2 DNA binding through autocrine IGF1/IGFR signaling to promote endothelial cell migration, proliferation, and angiogenesis [22] indicating glucose has a function in modulating RUNX2 activity. Hyperglycemic conditions, however, inhibited RUNX2 activity through the aldose reductase polyol pathway [22]. Inhibition of RUNX2 by hyperglycemia inhibited endothelial cell migration, proliferation, and angiogenesis [20, 22]. Treatment with 2-deoxyglucose (inhibitor of glucose metabolism) under euglycemic growth conditions in endothelial cells resulted in a delayed exit from G1/S into G2 phases of the cell cycle with subsequent lower levels of RUNX2 DNA binding activity [20]. Similarly, nutrient and serum deprivation blocked endothelial cell exit from G1 [20]. Using shRNA lentiviral knockdown to reduce RUNX2 levels, exit from G1 and progression through the cell cycle was found to be dependent upon RUNX2 [20].

3.4.2 Regulation of RUNX2 Expression

RUNX2 expression in osteoblasts promotes differentiation through tight control of RUNX2 activity to maintain the balance of bone formation and remodeling. Similarly, during tumorigenesis many of the negative regulators of RUNX2 are inhibited, thus allowing sustained expression and activity. In general, RUNX2 expression and DNA-binding activity depend on transcriptional mechanisms of activation versus repression and on post-translational protein modifications that include phosphorylation, acetylation, and ubiquitination [74].

3.4.2.1 Transcriptional Activation and Repression

Osteoblast differentiation requires activation of RUNX2, but what regulates RUNX2 expression in pre-osteoblasts has remained an enigma for quite some time. Tu et al. determined that activation of RUNX2 expression is dependent upon Indian hedgehog (Ihh) signaling [75]. In Ihh null mice even the forced expression of RUNX2 failed to induce osteoblast differentiation [75] confirming that Ihh is not only important for activation of RUNX2 expression but also for RUNX2 activity. In osteoblasts, it was found that BMP-2 increased RUNX2 induction and expression [76]. Shu et al. generated BMP-2 knockout mice and found a dramatic reduction in RUNX2 expression resulting in severe chondrodysplasia [76]. BMP-2 was also able to upregulate PlexinA2 (PlxnA2) in pre-osteoblastic cells [77]. The upregulation of PlxnA2 was associated with increased RUNX2 expression, osteoblast differentiation, and bone mineralization [77]. This upregulation of RUNX2 was thought to be a result of PlxnA2 stabilization of BMP-2 binding to BMP-2 receptors.

TWIST, a transcription factor implicated in epithelial-mesenchymal-transition (EMT) and metastasis in many types of cancers is also an essential transcription factor in development. TWIST was shown to be an inhibitor of RUNX2 expression in bone marrow-derived mesenchymal stem cells [78]. Under low oxygen conditions (hypoxia), Hif1α upregulated expression of TWIST, which directly inhibited RUNX2 expression [78] and osteoblast differentiation. Conversely, studies in PC3 prostate cancer cells showed that TWIST enhances RUNX2 expression levels to promote metastasis to the bone [79]. Glucocorticoid receptor binding to the P2 promoter of RUNX2 was shown to inhibit RUNX2 expression [80]. This resulted in adipocyte differentiation [80] instead of osteoblast differentiation and reveals how signaling events can lead to cell fate determination by regulating RUNX2 expression.

RUNX2 expression is increased in breast tumors where it has been implicated in mediating metastasis to the bone. How RUNX2 expression is enhanced in breast cancer tissue is poorly understood. Using breast cancer cells, one group was able to show that serotonin induced parathyroid hormone related protein (PTHrP), which in turn increased transcription of RUNX2 [40]. Since PTHrP is also a RUNX2 target gene, it fuels a feed forward loop potentiating maximal RUNX2 expression to promote progression of breast cancer to a metastatic stage. In multiple myeloma (MM) it has been shown that Gfi1 is upregulated and represses RUNX2 expression [81]. This inhibition of RUNX2 then results in inhibition of osteoblast differentiation.

3.4.2.2 Post-translational Regulation

Intracellular signaling is able to modulate RUNX2 post-translational modifications to regulate DNA-binding activity and RUNX2 levels. Using antibodies to detect phosphoserine sites on RUNX2 in endothelial cells, it was shown that RUNX2 is phosphorylated under euglycemic growth conditions by cyclin dependent kinase 4 (cdk4). This phosphorylation was abrogated by mutation of the cdk site S451 [20].

A RUNX2-S451A mutant showed inhibition of DNA binding in endothelial cells as well as a reduction in wound healing activity [20]. Inhibition of cdk4 produced similar results demonstrating that cdk4 can activate RUNX2 through phosphorylation of S451 in response to glucose. In addition to glucose modulation, RUNX2 activity was cell cycle regulated [136, 137]. RUNX2 was associated with DNA when cells were proliferating but was sequestered to subnuclear loci when cells were quiescent [20, 23, 136]. RUNX2 protein levels were maximal in endothelial cells in late G2 and M phases of the cell cycle [23]. Using RNA interference to knockdown RUNX2, endothelial cell exit from G2/M phases of the cell cycle was delayed [23] resulting in a decrease in cell proliferation. In vitro kinase assays showed S451 must be phosphorylated to allow RUNX2 to function in promoting progression through the cell cycle [23]. However, in osteoblasts, RUNX2 inhibited osteoblast proliferation and RUNX2 protein levels were maximal in G1 [24], suggesting that RUNX2 regulation may be cell type dependent.

BMP2 is able to regulate RUNX2 protein levels via inhibition of cdk4 [76]. This inhibition leads to protection from proteasomal degradation thus maintaining cellular protein levels. Recent research has focused on how micro-RNA's (miRNAs) regulate RUNX2 protein levels. MiRNAs modulate protein levels through an RNA interference pathway ultimately leading to mRNA degradation or reduced protein translation. MiRNAs are 18–25 nucleotide RNAs that repress translational activity of mRNAs [82]. Wu et al. have suggested that the miR-30 family of miRNAs may play an essential role in osteogenesis. Their data show that miR-30 was able to negatively regulate both Smad1 and RUNX2 [82]. Alkaline phosphatase (marker of osteoblast differentiation) was shown to be dramatically decreased after exogenous miR-30 expression [82]. Furthermore, miR-30 family miRNAs were able to bind to the 3′-untranslated region of both Smad1 and RUNX2 mRNA [82] thus inhibiting the effects of BMP-2-stimulated osteoblast differentiation pathways. MiR-203 is a known tumor suppressor miRNA which is downregulated in prostate cancer [83, 84]. It has been shown to bind RUNX2 mRNA resulting in a mesenchymal to epithelial transition (MET), inhibition of cell proliferation, and inhibition of cell migration and invasion [83, 84]. In addition to the miRNAs already described, it has been shown that miR-23a, miR-34 cluster, miR-133a, miR-135a, miR-137, miR-204, miR-205, miR-217, miR-218, and miR-338 all regulate RUNX2 expression [85, 86]. In addition to miRNA regulation of RUNX2, the proteasomal degradation pathway is implicated in regulating RUNX2 levels in the cell. For example, WWP1 (WW domain-containing E3 ubiquitin protein ligase 1) has been shown to function as the E3 ubiquitin ligase responsible for ubiquitinating RUNX2 and targeting it for proteasomal degradation [87].

3.5 RUNX2 as a Therapeutic Target

RUNX2 is a transcriptional regulator of gene expression. Mutations, amplification, or inappropriate expression of RUNX2 has the potential to amplify the expression or repression of a variety of target genes. This could regulate global changes in gene

regulatory networks and lead to a process called transformation amplification. Therefore, RUNX2 may be a prime target for therapeutic intervention [88, 128] to treat disease because multiple transformation pathways could be inhibited. Many cancers develop resistance to therapies due to signaling pathway redundancy allowing signaling switches to occur. Being able to target transcription factors allows therapies to bypass the redundancy of signaling.

Prostate cancer bone metastases form osteolytic lesions before the development of osteoblastic lesions. Li et al. found that isoflavone and 3, 3′-diindolylmethone (BR-DIM) are able to multifunctionally inhibit these metastases from forming [88]. This combination therapy may inhibit not only osteoblast differentiation but also osteoclast differentiation. Their research showed that one of the ways in which this combination treatment functions is to inhibit signaling of RANKL [88]. However, further cellular analysis revealed isoflavone and BR-DIM combination therapy inhibited signaling from the Akt, AR (androgen receptor), PSA, and p27 signaling axis as well as blocking the RNA interference pathway by inhibiting miR-92a which is associated with RANKL signaling [88]. This is one example of inhibiting the effects of RUNX2 through targeting of its target genes.

Natural compounds have been shown to have potent medicinal benefits. Astragaloside II a compound from the plant, *Radix astragalus*, was tested on rat primary osteoblasts to determine its effects on viability, proliferation, differentiation and maturation [89]. Astragaloside II promoted proliferation, differentiation and mineralization of primary rat osteoblasts [89]. The effects of this drug on post-menopausal women could potentially prevent osteoporosis. One benefit in cancer patients could be prevention of bone fractures associated with osteolytic metastases by promoting osteoblast differentiation to prevent bone degradation caused by cancer cells. However, use of this drug for patients with metastatic cancers could increase the incidence of bone metastases and lead to early death by stimulating RUNX2 expression within tumor cells. A second compound, Neobavaisoflavone (NBIF), was isolated from the plant *Psoralea corylifolia L* and was shown to have a similar effect on RUNX2 in osteoblasts as Astragaloside II [90]. NBIF was shown to upregulate RUNX2 expression in MC3T3-E1 cells while also activating its gene regulatory functions [90]. NBIF was shown to upregulate osteocalcin, bone sialoprotein, and type 1 collagen [90]. While this drug may have pro-bone forming functions and could potentially be a way to restore bone loss as a result of bone degradative diseases, one must also take into consideration the dosing regimen that would make this drug specific for osteoblasts while not further stabilizing RUNX2 positive cancer cells.

An understanding of upstream signaling pathways that activate RUNX2 and how cofactors regulate RUNX2 activity in disease would improve the development of therapeutics. In MM it was shown that the Gfi1 targeted drug, Trichostatin-A, was able to block the inhibition that Gfi1 imposes on RUNX2 [81]. Bortezomib (Velcade) is a proteasomal inhibitor and is also used in the treatment of MM because it was shown to induce osteoblast differentiation [91]. Bortezomib inhibited FGF2 induced TAZ (a RUNX2 binding coactivator) protein degradation [91], thus allowing TAZ to interact with RUNX2 and promote osteoblast differentiation in MC3T3-E1 cells. Therefore, restoration of RUNX2 expression enabled osteoblast differentiation and disabled MM progression in the bone microenvironment.

Studies that show targeting upstream signaling pathways that activate RUNX2 or cofactors have been reported, but the research for direct RUNX2 inhibition is limited. Our laboratory showed for the first time that cholecalciferol (inactive Vitamin D3 precursor) directly modulates RUNX2 DNA-binding activity [25]. Cholecalciferol is produced in the skin as a result of exposure to UV or it can be absorbed in the digestive system through the diet [25]. Cholecalciferol is normally converted to active Vitamin D3 in the body to 1,25OH-D3, which interacts with Vitamin D Receptor (VDR) to promote calcium absorption in the gut and increase bone formation. However, Vitamin D3 also exhibits paracrine and autocrine activity by regulating epithelial cell differentiation and modulating immune system function [25]. Using a quantitative DNA binding assay (D-ELISA) it was shown that cholecalciferol was able to modulate RUNX2 DNA binding in a VDR-independent manner. Analysis of RUNX2-positive breast tumor cells (MCF7), endothelial cells (HBME), and osteosarcoma cells (SaOs2) showed that cholecalciferol was able to inhibit cellular proliferation [25], suggesting a RUNX2-specific function. Further research needs to be conducted to study the effects in animal models before this strategy could be modified for clinical trials in the treatment of RUNX2 positive tumors.

Direct targeting of epithelial or bone cell RUNX2 pathways and cofactors that modulate RUNX2 expression or activity is another therapeutic strategy. However, targeting the microenvironment is also another option. Angiogenesis is an essential process that must occur for many solid tumors to metastasize to bone. Endothelial cells express RUNX2, which mediates wound healing by stimulating new blood vessel formation. However, tumors use angiogenesis to vascularize and provide nutrients to tumors that also allow cancer cells to intravasate. Inhibition of angiogenesis using VEGF inhibitors has been a therapeutic strategy for many years. In theory if one starves tumors of their nutrient supply, it should lead to tumor regression or necrosis. Alternatively, by stabilizing blood vessels feeding the tumors then in theory chemotherapeutic agents would be able to get to sites of tumor growth more effectively. However, these therapies have been less successful in the clinic. LGD1069 is a selective retinoid X receptor ligand used to treat T-cell lymphoma but has also been shown to inhibit angiogenesis in lung cancer [92]. LGD1069 inhibited activation of the TGF-β/Smad pathway thus reducing both activation and expression of RUNX2 in human umbilical vein endothelial cells (HUVECs) [92]. However, endothelial cells are not the only cells that use the TGF-β/Smad pathway to activate the expression and activity of RUNX2. Therefore, LGD1069 activity in other cell lines (breast, prostate, and osteosarcoma) should be tested to determine whether it decreases RUNX2 protein or activity. In melanoma, it was shown that SD-208 (a TGF-β receptor I kinase inhibitor) blocked expression of RUNX2 through downregulation of TGF-β/Smad signaling [93], supporting the notion that targeting of the TGF-β/Smad axis could have therapeutic benefit in RUNX2 positive tumors by inhibiting expression within the tumor itself or by preventing angiogenesis.

3.6 Future Directions

The biological understanding of disease will be essential to creating new therapeutics to treat disease. Signaling pathways are difficult to ablate because of pathway redundancy. Therefore, it is essential to target upstream signaling as well as downstream signaling, especially of transcription factors. Understanding the role that RUNX2 plays in cancer progression will be essential to be able to use it as a therapeutic target to inhibit metastasis to the bone. RUNX2 has been shown to be highly upregulated in the cancer stem cell (CSC) populations of prostate cancer, breast cancer, and osteosarcoma [94–98]. Therefore, with the emergence of the CSC as a key contributor to cancer development, progression, and resistance to modern therapies, it will be important to understand the role that RUNX2 is playing in CSC regulation so that appropriate therapeutic strategies can be developed.

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Chapter 4
Epigenetic Mechanisms of Cancer Metastasis

Jing Liang and Yongfeng Shang

Abstract Metastasis refers to the process that cancer cells leave their primary tumor mass, break into blood and lymphatic vessels, and travel to distant organ sites throughout the body where they may establish new colonies. Metastasis is responsible for 90 % of cancer mortality. Increasing evidence suggest that epigenetic mechanisms, such as DNA methylation and histone modifications, play an important role in mediating the invasion-metastasis cascade. Targeting deregulated epigenetic modification enzymes by small-molecule inhibitors is a promising therapeutic strategy for the treatment of metastatic cancers.

Keywords Epithelial-mesenchymal transition (EMT) • Cancer metastasis • DNA methylation • Histone modification • Epigenetic therapy

Abbreviations

DNMT	DNA methyltransferase
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition

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HATs	Histone acetyltransferases
HDACi	HDAC inhibitors
HDACs	Histone deacetylases
MET	Mesenchymal-epithelial transition
MMPs	Matrix metalloproteinases
PTMs	Post-translational modifications

Cancer is characterized by uncontrolled growth and spread of abnormal cells. Primary tumors do not necessarily cause obvious discomfort to the patient, simply because some organs are well expansible while maintaining their normal functions. A primary tumor in the breast will not cause much trouble to the patient's overall physiological function, unless breastfeeding is needed. Only 10 % of deaths from cancer are due to primary tumors, while metastasis is responsible for the remaining 90 % of cancer mortality. Metastasis refers to the process that cancer cells leave their primary tumor mass, break into blood and lymphatic vessels, and travel to distant organ sites throughout the body where they may establish new colonies. Metastases of breast cancer can be found in the brain, liver, bones, and lungs. Insidious growth of metastatic cancer cells in these sites is life-threatening because the physiological functions of such vital organs are greatly compromised.

While the molecular mechanisms underlying tumorigenesis have been studied in great detail, our understanding of cancer metastasis is limited, partly due to the difficulty to set up the in vitro and in vivo experimental models for this process. Currently, cancer metastasis is considered to be a cascade that consists of a series of interrelated steps, including (i) detachment of tumor cells from the primary tumor; (ii) invasion into the surrounding tissues; (iii) intravasation into the blood or lymphatic vessels; (iv) dissemination in the blood stream or the lymphatic system and, finally, (v) extravasation and colonization at a secondary site (Fig. 4.1) [1, 2]. While certain genetic lesions provide primary tumor cells good opportunity for successful metastasis, the interplay between tumor cells and the surrounding stroma eventually endow tumor cells the ability to fulfill these daunting tasks. Epigenetic mechanisms, including DNA methylation, histone modification, nucleosome remodeling, and RNA-mediated targeting, alter gene expression profile via changes in the chromatin states. The epigenome of cells is dynamic in response to extra- and intracellular signals, while under certain circumstances it can also be transmitted to the next generation of cells to maintain cell identity. Increasing evidence suggest that epigenetic mechanisms play important roles in tumor progression and metastasis. In this chapter, we first describe our current understanding of the major molecular events governing the invasion-metastasis cascade, and then discuss how epigenetic factors, particularly DNA methylation and histone modifications, contribute to the metastasis process.

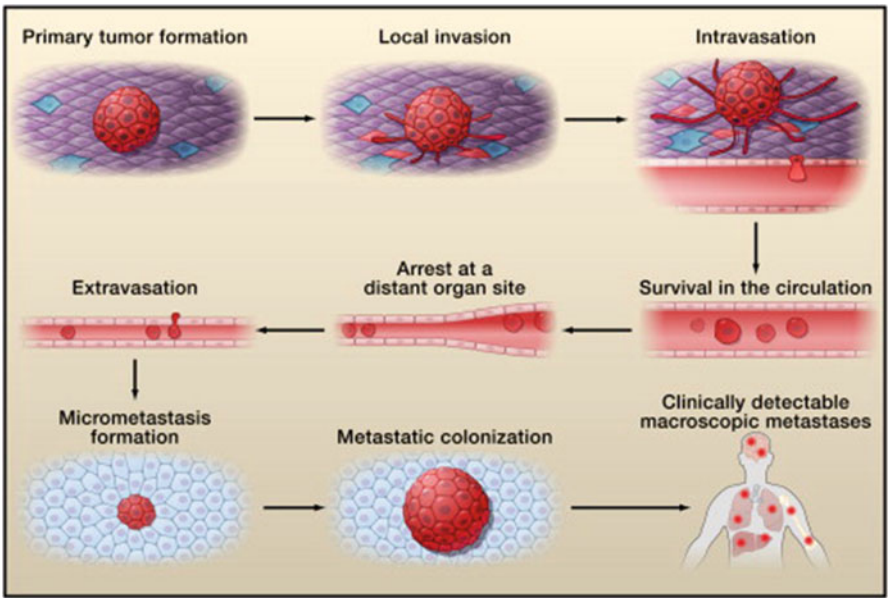

Fig. 4.1 The invasion-metastasis cascade. During metastatic progression, tumor cells exit their primary sites of growth, invade into the extracellular matrix, and enter into the blood or lymphatic vessels (local invasion, intravasation). Circulating cancer cells translocate systemically (survival in the circulation, arrest at a distant organ site, extravasation), and adapt to survive and thrive in the foreign microenvironments of distant tissues (micrometastasis formation, metastatic colonization). Carcinoma cells are depicted in *red* (From S. Valastyan and R.A. Weinberg. Tumor Metastasis: Molecular insights and evolving paradigms. *Cell* 147: 275–292, 2011. Reprinted with kind permission from Elsevier Limited)

4.1 The Epithelial-Mesenchymal Transition Enables Carcinoma Cells to Become Invasive and Represents the Initial Step of Metastasis

Malignant cells arise from epithelial tissues form carcinomas, which contribute to the majority of life-threatening cancers. Early stage carcinoma cells retain the typical epithelial property, which includes lateral tight connection by specialized junction structures, aligned apical-basal polarity through association with the basement membrane, and lack of cell motility. Later on, many of these cancer cells acquire the ability to invade the nearby stroma, travel throughout the body via the lymphatic or hematogenous circulation, and form disseminated colonies in distant organs. To understand how epigenetic mechanisms play a role in cancer metastasis, we need to dissect the process into sequential biological steps, with the notion that these steps are interrelated to each other in reality.

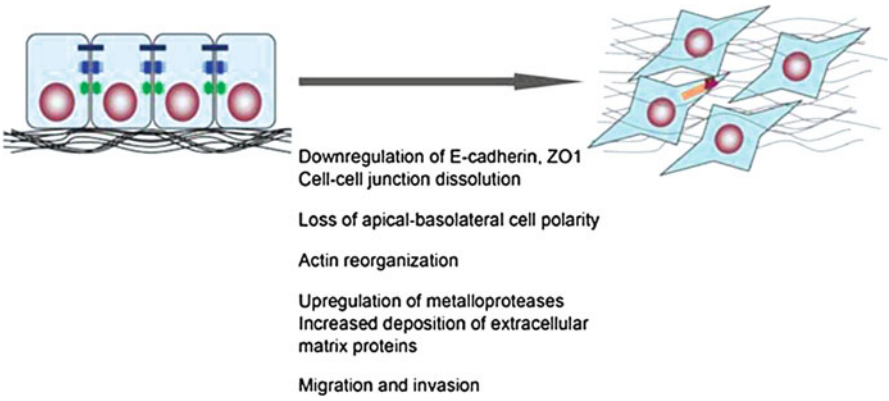

Fig. 4.2 Epithelial-mesenchymal transition. Epithelial-mesenchymal transition (EMT) occurs when epithelial cells lose their epithelial cell characteristics, including dissolution of cell-cell junctions, i.e. tight junctions (*black*), adherens junctions (*blue*) and desmosomes (*green*), and loss of apical-basolateral polarity, and acquire a mesenchymal phenotype, characterized by actin reorganization and stress fiber formation (*red*), migration and invasion (From J. Xu, S. Lamouille and R. Derynck. TGF-[beta]-induced epithelial to mesenchymal transition. *Cell Res* 19: 156–172, 2009. Reprinted with permission from Nature Publishing Group)

As long as tumor cells are separated from the surrounding tissues by an intact basement membrane, they are not truly "malignant". Once in situ carcinoma cells breach the basement membrane and invade into the stroma, the initial step of metastasis is manifested. Arise from epithelial tissues, original carcinoma cells has poor motility, and the lateral cell-cell connection and the apical-basal polarity do not allow these cells to invade through the underlying extracellular matrix (ECM). To initiate the first step of metastasis, carcinoma cells must transform someway to shed these epithelial properties that suppress invasion, and acquire mesenchymal phenotypes such as better motility, loose cell-cell contacts, and affinity to the stroma. The epithelial-mesenchymal transition (EMT) is now generally accepted to be the critical initial step of cancer metastasis (Fig. 4.2).

EMT was recognized as a feature of embryogenesis in the early 1980s. One example of EMT in embryonic development is the formation of the mesoderm, the precursor of mesenchymal tissues, during gastrulation in early embryogenesis. At this stage, ectoderm cells, which are located at outer side of the embryo and arrayed in an epithelial cell layer, migrate inward toward the center of the embryo to form the mesoderm, where fibroblasts and hematopoietic cells originate. EMT can also be witnessed in wound healing, in which process the epithelial cells at the edge of a wound acquire mesenchymal phenotype and become motile and invasive to fill in the gap in the epithelium created by the wounding. Therefore, EMT is not a unique nature of carcinoma cells; rather it is the activation of a reprogramming behavior that is usually confined to early embryogenesis and wound healing in adult.

Fundamental alterations of gene expression lead to reconstituted cellular machinery, and ultimately result in the phenotypic changes of EMT cells. Among all the molecules that influence epithelial versus mesenchymal phenotypes, the epithelial specific E-cadherin, encoded by *CDH1* gene, plays the dominant role. E-cadherin is a transmembrane glycoprotein, and the cytoplasmic domains of E-cadherin are tethered to the actin fibers of the cytoskeleton via a complex of α- and β-catenins. E-cadherin molecules are displayed on adjacent epithelial cells and tether the apposed plasma membranes to one another. Loss of E-cadherin is consistently observed at the sites of EMT. Epithelial originated cells acquire a mesenchymal morphology and increased motility when the expression of E-cadherin is suppressed, whereas re-expression of this protein in invasive cancer cells strongly suppressed their metastatic dissemination. Besides E-cadherin, loss of expression of other epithelial specific proteins, such as α-, β-, and γ-catenins, and gain of expression of mesenchymal markers, such as fibronectin, vimentin, N-cadherin, is constantly observed during EMT [3, 4].

4.2 The Interplay Between Cancer Cells and the Stroma Provide Signals for the Epithelial-Mesenchymal Transition

EMT is an induced, reversible process, as carcinoma cells often revert back to a more epithelial phenotype once these cells reach the distant organs, where EMT-inducing signals are no longer existed. Therefore, the metastases resemble the phenotype of the primary carcinomas. This process is often called mesenchymal-epithelial transition (MET). EMT can be induced or regulated by various growth and differentiation factors, including TGF-β (transforming growth factor-β), TNF-α (tumor necrosis factor- α), EGF (epidermal growth factor), HGF (hepatocyte growth factor), and IGF-1 (insulin-like growth factor-1). These heterotypic signals are mainly released by the stroma of primary carcinomas, and cells located at the outer edges of the neoplasm sense these signal and undergo EMT. Among these factors, the major inducer of EMT is TGF-β, which can also produced by cancer cells themselves to generate a positive feedback loop for EMT induction. Culturing EpRas tumor cells in the presence of TGF-β, these epithelial, cobblestone-like cells change to an elongated fibroblastic phenotype. The levels of tumor-associated TGF-β (often TGF-β1) were frequently found to be correlated with increasing degrees of tumor invasiveness. High levels of TGF-β in the blood are often predictive of poor prognosis for the cancer patient. EMT-inducing growth factors activate different intracellular signaling cascades in carcinoma cells, such as NF-κB and Wnt pathways, leading to the alteration of gene expression profiles that favor mesenchymal phenotypes.

In response to various inductive signals, EMT-inducing transcription factors can serve as major signaling mediators of the EMT program to promote metastasis. Ectopic expression of these transcription factors enables epithelial cells undergo

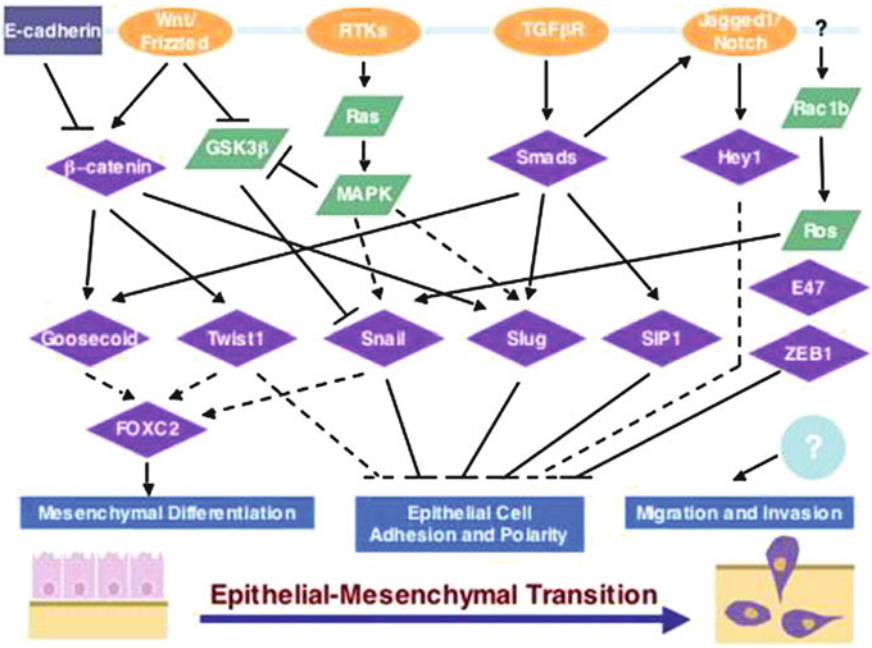

Fig. 4.3 Signaling pathways and transcription factors that regulate the epithelial-mesenchymal transition in carcinoma cells. In cancer cells, the TGF-β signaling pathway induces multiple EMT-inducing transcription factors, including Slug, SIP1, and Goosecoid, via activation of Smads. The Wnt pathway and loss of E-cadherin from adherens junctions activate β-catenin, which in turn induces several EMT-inducing transcription factors as well, such as Slug, Twist1, and Goosecoid. Multiple tyrosine kinase receptor (TKR) pathways, including FGFR, EGFR, PDGFR, and HGFR, can induce the expression of Snail and Slug through the Ras-MAPK pathway. Among all the EMT-inducing transcription factors, Snail, Slug, SIP1, and E47 directly suppress E-cadherin transcription, while Twist1, Goosecoid, and FOXC2 seem to function indirectly. FOXC2 is induced in tumor cells expressing Twist1, Snail, and Goosecoid and mediates mesenchymal differentiation. *Solid lines* indicate direct transcriptional or posttranscriptional regulations. *Dashed lines* indicate indirect regulation (From J. Yang and R.A. Weinberg. Epithelial-mesenchymal transition: At the crossroads of development and tumor metastasis. *Dev Cell* 14: 818–829, 2008. Reprinted with kind permission from Elsevier Limited)

EMT and acquire mesenchymal phenotype (Fig. 4.3). Examples of these EMT-inducing transcription factors include Twist, Snail, Slug, ZEB2/ZFXH1B/SIP1, and Goosecoid. These proteins are often highly expressed during embryogenesis, and their reactivation allows carcinoma cells to initiate the EMT program. Accumulating evidence associates these transcription factors with various malignancies, especially those with highly invasive behavior and poor prognosis. These transcription factors can regulate a panel of downstream target genes that collaboratively program an EMT process. For example, the zinc-finger transcription factors Snail, Slug, ZEB1 and ZEB2 are capable of directly repressing the transcription of E-cadherin and several polarity factors, including Crumbs3 and Lgl2 [5, 6].

4.3 Disseminated Cancer Cells Found Metastases in Distant Organs

Once primary tumor cells undergo EMT, they acquire abilities for further invasion. Before they enter the general circulation, EMT-transformed tumor cells first need to pave the way in the ECM by either dissolving the stroma or pushing aside any cells that stand in their path. Among the molecular events that govern this process, the best understood is the secretion of matrix metalloproteinases (MMPs), which is a class of secreted proteases that can degrade specific components of the ECM. MMPs are secreted by tumor-recruited stroma cells, such as macrophages, mast cells, and fibroblasts. Dissolving of the ECM by MMPs creates spaces for the tumor cells to move. MMPs also mobilize and activate some growth factors that have been tethered in the inactive form to the ECM or to the cell surface.

Motile tumor cells adhere to the vessels of blood or lymphatic systems in the stroma and enter the general circulation, a process that is often referred to intravasation. Before these cells reach their destination, they may experience much ordeal such as mechanical shearing forces of the blood stream and attack from immune cells in the circulation. The molecular mechanisms that govern these events are largely unknown. Theoretically, metastatic cancer cells can disseminate all over the body, but several organs, such as the lung, bone, liver, and brain are clearly more prone for metastases to grow. In addition, subtypes of tumor cells from different tissue origin have their own preference of metastatic organs. The vast majority of cells that end up forming small micrometastases (<2 mm diameter) never succeed in growing into macrometastases (>2 mm diameter) that are clinically relevant for the patients' health. In this sense, colonization is extremely inefficient, and often considered to be the rate-limiting step in the entire invasion-metastasis cascade. Although the detailed molecular mechanisms are yet to be clarified, it is important to understand that it is the complex interplay between metastatic cancer cells and the microenvironment of a foreign tissue that essentially determines whether colonization will be successful and metastatic diseases will ever develop. Dispersed cancer cells will only survive and found colonies in the environment with appropriate chemokines, trophic factors, and mitogens, while seeding cancer cells also release heterotypic signals to reshape the landed tissues. In this adaptation process, gene expression reprogramming in the metastatic cancer cells is inevitable.

Analogous to oncogenes and tumor suppressor genes that regulate tumorigenesis, there are many genes that function positively or negatively in the regulation of metastasis. Metastasis promoting regulators can be growth factors, growth factor receptors, transcription factors, or key components in signal transduction pathways. When genes encode these molecules are ectopically expressed in epithelial cells, they are able to elicit metastasis-prone phenotypic changes, such as increased cell motility and decreased cell polarity. On the other hand, metastasis suppressor genes encode proteins that specifically inhibit invasion and metastasis without affecting the growth of primary tumors. To date, over 20 metastasis suppressor genes have been identified, with the best characterized is *CDH1*, which encodes the

epithelial-specific E-cadherin. Although the molecular functions of many metastasis suppressor genes are yet to be clarified, altered expression of these genes, whether due to genetic defects or epigenetic regulations, can significantly affect the overall invasion-metastasis process [7].

4.4 Promoter Methylation is an Important Epigenetic Mechanism to Regulate the Expression of Metastasis Related Genes

The long journey of invasion-metastasis consists of multiple steps that involve the interplay between primary tumor cells and the surrounding cells in the stroma, blood, and the distant foreign tissues. From the initial EMT to the final colonization, cancer cells constantly transduce heterotypic extracellular signals into changes of gene expression and cell behavior to better adapt themselves to metastasis. Unlike genetic defects such as gene amplification, deletion, or mutations, epigenetic regulations, such as chemical modifications of DNA sequences or histone proteins, are more flexible in response to various extracellular stimuli. In the mean time, certain epigenetic modifications can also be inherited by the next generation of cells to maintain relatively stable cell characteristics. In recent years, it is increasingly realized that epigenetic mechanisms play a profound role in the regulation of cancer metastasis. Epigenetics is commonly used to describe chromatin-based events that regulate DNA-templated processes. Direct DNA methylation at cytosine on CpG sequences, post-translational modifications (PTMs) of histones, the presence of histone variants, remodeling of nucleosomes, and non-coding RNA mediated targeting are the major epigenetic pathways that regulate many important biological processes [8]. In this chapter, we mainly focus on how DNA methylation and histone modifications play a role in the invasion-metastasis cascade.

Promoter cytosine methylation in CpG dinucleotides inactivates gene expression and can profoundly affect the metastasis cascade. DNA methylation is catalyzed by DNA methyltransferase (DNMT) enzymes. DNMT-1 is responsible for DNA methylation maintenance through its action on semimethylated CpG substrates. DNMT-3A and -3B are the *de novo* methyltransferases to newly methylate cytosine during early embryogenesis [9]. Promoter hypermethylation of certain metastatic suppressor genes is constantly observed in various invasive malignancies. Hypermethylation of the *CDH1* promoter in cancers leads to the loss of E-cadherin expression during cancer progression. A large CpG island in the 5′ proximal promoter region of the *CDH1* gene shows aberrant DNA methylation in many different human carcinomas and correlates with reduced E-cadherin protein expression. Exposure of cancer cells with demethylating agent 5′-aza-2′-deoxycytidine (5Aza-dC) reactivates E-cadherin expression in many cancer cell lines, leading to increased cell aggregation and reduced cell motility and invasiveness [6]. Kisspeptin (KISS-1) gene has been identified as a metastasis suppressor gene in various human

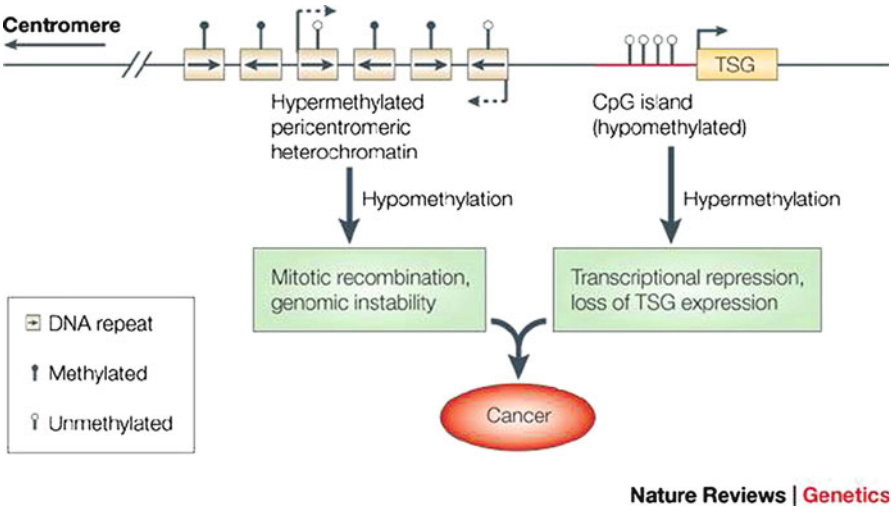

Fig. 4.4 DNA methylation and cancer. In normal cells, the repeat-rich, pericentromeric heterochromatin is hypermethylated and is transcriptionally silent. Actively transcribed tumour suppressor gene (*TSG*) is associated with a hypomethylated CpG island (indicated in *red*). In tumour cells, repeat-rich heterochromatin becomes hypomethylated and this contributes to genomic instability through increased mitotic recombination events. De novo methylation of CpG islands in the TSG or the metastasis suppressor gene can result in the transcriptional silencing of these genes, leading to unrestrained growth and spreading of the cancer cells (From K.D. Robertson. DNA methylation and human disease. *Nat Rev Genet* 6: 597–610, 2005. Reprinted with permission from Nature Publishing Group)

malignancies. KISS-1encodes a number of peptides (kp-54, kp-14, kp-13, kp-10), which are endogenous ligands to a G protein-coupled receptor called GPR54. The molecular basis of anti-metastatic activity of KISS-1 is not fully understood. Some evidences suggest that kisspeptin/GPR54 system negatively regulates MMP-9, a member of matrix metalloproteinases that degrade ECM. Secretion of KISS1 has also been demonstrated to be necessary to maintain dormancy in disseminated cancer cells, thus blocking metastatic colonization. Inactivation of KISS-1 gene by promoter hypermethylation has been observed in many invasive cancers such as melanoma, bladder cancer, and gastric carcinoma [10].

Genome-wide hypomethylation is a common feature of many malignant cells (Fig. 4.4). Hypomethylation of regulatory DNA sequences leads to overactivation of some oncogenes or EMT-inducing transcription factors, and general hypomethylation in heterochromatin regions is more deleterious as it results in genomic instability and subsequent multiple genetic defects [11]. Within a breast tumor mass, heterotypic cancer cells can often be sorted to two groups based on their phenotype and specific cell surface markers, namely stem cell-like, more invasive $CD44^{+}$ $CD24^{-}$cells and epithelial, more differentiated $CD44^{-}CD24^{+}$ cells. Promoter hypomethylation and the resultant overexpression of several EMT-inducing transcription factors have been observed in invasive $CD44^{+}$ $CD24^{-}$cells compare to their differentiated $CD44^{-}CD24^{+}$ counterparts [12].

4.5 Complex Post-Translational Histone Modifications Coordinately Regulate the Expression of Metastasis-Related Genes

In most cases, the role of promoter DNA methylation in gene regulation is relatively straightforward, in that hypermethylation inhibits gene expression whereas hypomethylation activates gene expression. By contrast, the influence of post-translational histone modifications on gene expression is far more complex and context-dependent. In eukaryotic cells, DNA is packaged into chromatin. The nucleosome is the fundamental unit of chromatin and it is composed of an octamer of the four core histones (H3, H4, H2A, H2B) around which 147 base pairs of DNA are wrapped. The core histones are predominantly globular except for their N-terminal "tails", which are unstructured and protrude from the globular core. Various post-translational chemical modifications, such as acetylation, phosphorylation, poly ADP-ribosylation, ubiquitination, and methylation, particularly those on the N-terminal tails, can alone or in combination affect gene transcription through direct remodeling of chromatin structure or recruitment of regulatory non-histone proteins (Fig. 4.5). For one particular histone modification, there are "writers" to catalyze the addition and "erasers" responsible for the removal of the mark. In addition, "readers" are those factors that bind to the specific modifications and respond to the "histone code" information conveyed by upstream signaling cascades. Aberrant histone modifications at either the candidate loci or genome-wide level, often caused by the deregulation of the writer/eraser/reader interplay, plays a significant role in mediating the invasion-metastasis cascade [13–15].

4.5.1 Histone Acetylation

Histone lysine acetylation neutralizes lysine's positive charge and consequently weakens the electrostatic interaction between histones and negatively charged DNA, leading to an "open" chromatin conformation in favor of transcriptional activation. In addition, acetylated lysine residues may serve as a "docking" site for non-histone proteins, which can carry their enzymatic activities and further modify chromatin. Histone acetylation is catalyzed by histone acetyltransferases (HATs) and is removed by histone deacetylases (HDACs). Usually, the catalytic activity of HATs and HDACs is not restricted to one particular lysine residue. Most of the identified human HATs, which mainly include three families, GNAT, MYST, and CBP/p300, function as transcriptional co-activators, and are recruited to chromatin by interacting with sequence-specific DNA-binding proteins [16]. For example, nuclear protein p300 acetylates multiple lysine residues on H3, H4, H2A, and H2B. p300 binds to transcription factor HNF3, and these two factors coordinately activate the transcription of *CDH1* gene, which encodes the metastasis suppressor E-cadherin. Ectopic expression of p300 in certain cancer cells can restore E-cadherin expression and repress their metastatic potential [17].

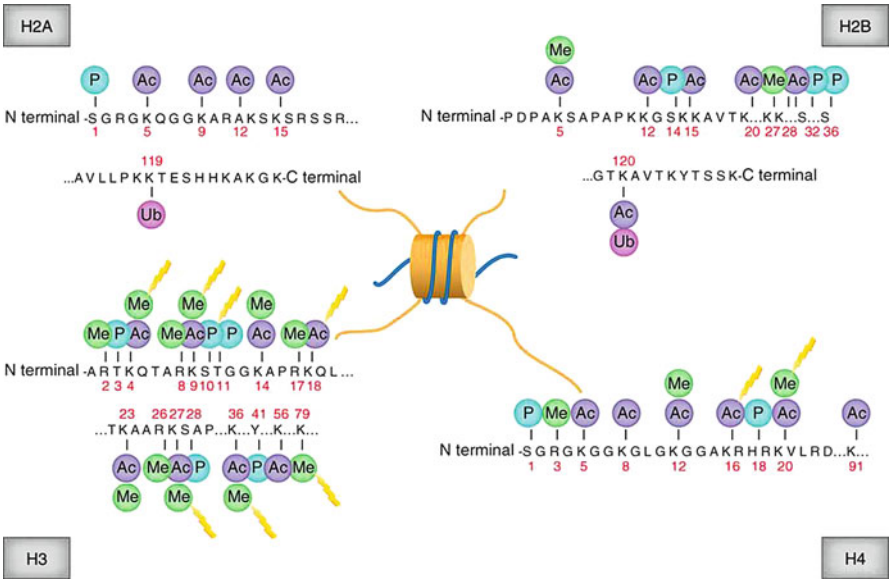

Fig. 4.5 Histone modification patterns in normal and cancer cells. Histones can undergo diverse post-translational modifications, especially on their protruding N-terminal tails. In the right combination and translated by the appropriate effectors, these modifications contribute to establishing the global and local condensed or decondensed chromatin states that eventually determine gene expression. This figure depicts the main modifications of the four core histones in normal cells (type and position in the amino acid sequence). Histone modifications typically associated with cancer have also been highlighted. *Ac* acetylation, *Me* methylation, *P* phosphorylation, *Ub* ubiquitination (From M. Rodríguez-Paredes and M. Esteller. Cancer epigenetics reaches mainstream oncology. *Nat Med* 17: 330–339, 2011. Reprinted with permission from Nature Publishing Group)

4.5.2 Histone Deacetylation

Deacetylation on lysine residues of histone proteins by HDACs are believed to cause chromosomal condensation and gene repression. There are three distinct families of histone deacetylases: the class I and class II histone deacetylases and the class III NAD-dependant enzymes of the Sir family. Class I HDACs include HDAC1, HDAC2, HDAC3, and HDAC8, and they are mainly localized to the nucleus. Class II HDACs include HDAC4-7, HDAC9, and HDAC10, and these proteins can shuttle between the cytoplasm and the nucleus. Class III HDACs are homologs of Sir2, a yeast transcriptional repressor that requires the cofactor NAD^+ for its deacetylase activity. Most of classes I HDACs are subunits of multiprotein nuclear complexes that are crucial for transcriptional repression [18]. At the E-cadherin promoter, EMT-inducing transcription factor Snail1 binds to the specific sequence called E-box elements, and recruits a repressive complex consisting of HDAC1, HDAC2 and SIN3A. Other EMT-inducing transcription factors Snail2/Slug, ZEB1, and ZEB2 similarly repress E-cadherin transcription by recruiting repressive protein complexes containing different class I HDACs [19].

4.5.3 Histone Methylation

Histone methylation occurs on all basic residues: arginines, lysines and histidines. The best-characterized sites of histone methylation are those that occur on lysine residues. The histone lysine methyltransferases and demethylases are responsible for addition or removal of methyl groups from different lysine residues on histones. These enzymes are highly specific, in that each enzyme regulates mono-, di-, or trimethylation of a single or a few lysine residues on histones. Because lysine methylation does not usually alter the charge of histone proteins, this modification per se has little influence on the interaction between histones and DNA. Instead, the location of the methyl-lysine residue on a histone tail and the degree of methylation (whether mono-, di-, or trimethylation) serve as the docking signal to recruit various reader proteins containing methyl-binding domains. To date, many methyl-binding domains have been identified. The classic methyl-binding domains are the Royal superfamily, including chromodomains, double chromodomains, chromobarrels, Tudor domains, double or tandem Tudor domains and the malignant brain tumor (MBT) repeats. Proteins with other domains, such as PHD finger, WD40 repeats, CW domains, PWWP domains, ankyrin repeats, have also been reported to recognize and bind to methylated lysine residues. Functionally, the combinations of the recruited regulatory proteins ultimately determine whether the transcription of local genes is activated or repressed [20].

The most extensively studied histone methylation sites include histone H3 lysine 4 (H3K4), H3K9, H3K27, H3K36, H3K79 and H4K20. The effects of methylation on gene transcription are diverse and context-dependent. In general, H3K4me3 is associated with active transcription or with genes that are poised for activation, whereas H3K27me3 is associated with repressed chromatin. H3K4me1 is often associated with enhancer function, whereas H3K4me3 is linked to promoter activity [21].

Aberrant histone methylation plays a role in cancer progression. Changes in global levels of certain histone methylation events are correlated with increased cancer recurrence and poor survival [20]. Mutations in or altered expression of histone methyltransferases correlate with various invasive cancers. These enzymes are often critical to the transcriptional regulation of key metastasis-related genes.

The SET-domain containing proteins and DOT1-like proteins have been shown to methylate lysine residues in histone and non-histone substrates. Enhancer of zeste homolog 2 (EZH2) contains a SET domain and is the catalytic component of the PRC2 complex, which is primarily responsible for catalyzing the trimethylation of histone H3 lysine 27 (H3K27me3). EZH2 has been found to be overexpressed in metastatic prostate cancer, and the expression level of EZH2 directly correlates with the aggressiveness of breast cancer. Ectopic expression of EZH2 in immortalized human mammary epithelial cell lines promotes anchorage-independent growth and cell invasion. In the molecular level, EZH2 usually acts as a transcriptional repressor through its H3K27me3 methyltransferase activity. Presumably, EZH2 promotes cancer metastasis by transcriptional inhibition of anti-metastasis genes. EZH2 is recruited to the promoter region of *CDH1* and represses E-cadherin expression in an

invasive prostate cancer cell line DU145 [22]. Interestingly, loss-of-function mutations in EZH2 gene confer a poor prognosis in certain hematopoietic malignancies, suggesting a tumor-suppressive role for EZH2 in these cell lineages [23, 24]. To date, the precise role of gain and loss of EZH2 activity in cancers and the underlying molecular mechanisms are an area of active investigation.

G9a (also known as KMT1C or EHMT2) catalyzes dimethylation of histone lysine 9 (H3K9me2). In TGF-β induced EMT cell line models, it has been demonstrated that transcription factor Snail recruits G9a and DNA methyltransferases to the *CDH1* promoter, leading to the inhibition of E-cadherin expression and the induction of EMT. Knockdown of G9a restores E-cadherin expression, inhibits cell migration and invasion, and reduces lung colonization of breast cancer metastasis [25].

SET8 (also known as PR-Set7/9, SETD8, KMT5A), a member of the SET domain-containing methyltransferase family, catalyzes monomethylation of H4K20. Recent studies indicate that SET8 and the transcription factor Twist are functionally interdependent to promote EMT and enhance the invasiveness of breast cancer cells in vitro and in vivo. SET8 is recruited to the *CDH1* promoter by Twist and repress E-cadherin expression; interestingly, SET8 and Twist are also present at the N-cadherin promoter, where they enhance the transcription of the gene. Together, SET8 and Twist coordinately promote EMT and cell invasiveness, and the dual function of SET8 at different promoters reinforce the notion the effect of histone methylation on gene transcription is context-dependent, relying on the combinations of histone modifications nearby and the distinct sets of regulatory proteins recruited [26].

4.5.4 Histone Demethylation

Two families of demethylases, the amine oxidases and jumonji C (JmjC)-domain-containing, iron-dependent dioxygenases, have been identified thus far to demethylate methyl-lysines [27]. Lysine-specific demethylase 1 (LSD1) is an amine oxidase that catalyzes the removal of mono- and di-methylation from histone H3 lysine 4 (H3K4). LSD1 regulates several intracellular signaling pathways including that of TGFβ1, which plays a critical role in cancer metastasis as we mentioned earlier in this chapter. In breast cancer tissue samples, the level of LSD1 is negatively correlated with that of TGFβ1. LSD1 downregulates TGFβ1 and inhibits the invasiveness and metastasis of breast cancer cells in vitro and in vivo [28]. Responsible for the removal of H3K4 trimethylation, JARID1B/PLU-1 belongs to the JmjC family of demethylases. Through its enzymatic activity, JARID1B removes the active H3K4me3 marks at the promoter regions of various genes, including CCL14, an epithelial derived chemokine. JARID1B inhibits the expression of CCL14 and suppresses the angiogenic and metastatic potential of breast cancer cells [29].

In many cases, multiple histone and DNA modification enzymes collaborate to regulate target gene expression. These factors may form a stable protein complex; alternatively they can assemble and dissemble dynamically at different chromatin

locations. H3K27me3 methyltransferase EZH2 physically interacts with DNA methyltransferases at the promoters of certain target genes, leading to the silence of these genes. The transcriptional repressive protein complex NuRD contains multiple components with different chromatin-related activities, and these components include chromatin remodeling factors (metastasis tumor antigen, MTA), histone deacetylases (HDAC1 and HDAC2), histone binding proteins (RbAp46 and RbAp48), methyl CpG-binding proteins (MBD2 and MBD3), DNA helicase/ATPase (Mi-2α/β), and in certain circumstances histone demethylases (LSD1 and JARID1B). Except for the core components, multiple NuRD subunits could be dynamically incorporated into the big protein complex at different chromatin locations, allowing more specific and fine-tuned regulation of different target genes [30, 31].

4.6 Epigenetic Therapy for Cancer Metastasis

Unlike genetic defects, epigenetic aberrations that lead to cancer metastasis are theoretically reversible, making them the ideal drug targets for cancer therapeutics. By inhibition of the particular modification enzymes that are responsible for the addition or removal of the chemical groups from DNA or histones, we may adjust the aberrant epigenome and the abnormal gene expression in cancer cells (Fig. 4.6). Although effective inhibitors of many DNA or histone modification enzymes have been developed, these epigenetic drugs have certain embedded problems that may limit their applications until we get better understanding of the molecular mechanisms of cancer epigenetics. To what degree an inhibitor is specific to a particular enzyme? To what degree inhibition of this enzyme is specific to regulate growth- and metastasis-associated genes in cancer cells? Does inhibition of epigenetic modification enzymes affect the biological function of non-cancer cells? These problems directly affect the efficacy and toxicity of the potential epigenetic anti-cancer drugs. Despite the above concerns and the lack of detailed molecular mechanisms, there are several epigenetic drugs that have been shown to effectively reverse metastatic phenotype in different cancer cell lines, and many clinical studies have evaluated specific enzyme inhibitors in the treatment of cancer metastasis [6, 32, 33].

As we have repeatedly stated earlier, the E-cadherin encoding gene *CDH1* is a critical metastasis suppression gene. Transcription of *CDH1* gene is silenced or reduced in many cancers with high metastasis potential. Reactivation of the *CDH1* gene expression is an important anti-metastasis therapeutic strategy. It has been demonstrated that inhibition of the activity of either DNMTs or histone modification enzymes that remove certain repressive histone modifications can effectively upregulate E-cadherin expression. Treatment of several invasive cancer cell lines with the DNMT inhibitor 5-aza-2′-deoxycytidine leads to restoration of E-cadherin expression and reversion of these cells to the epithelial phenotype. DNMT inhibitors 5-azacytidine and 2′-deoxy-5-azacytidine have been approved for the treatment of myelodysplastic syndrome, a pre-leukemic bone marrow disorder. It is important

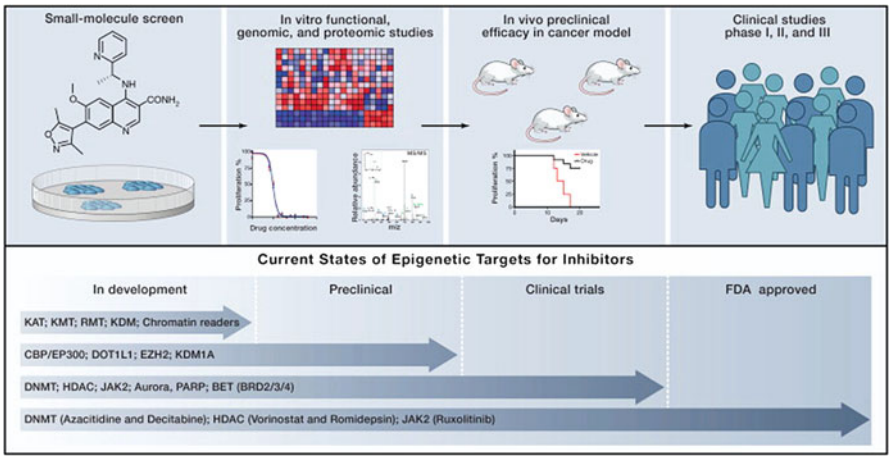

Fig. 4.6 The process to develop epigenetic drugs and the current status of various epigenetic therapies in cancer. Candidate small-molecule inhibitors are first tested in vitro in malignant cell lines for specificity and phenotypic response. These may, in the first instance, assess the inhibition of proliferation, induction of apoptosis, or cell-cycle arrest. These phenotypic assays are often coupled to genomic and proteomic methods to identify potential molecular mechanisms for the observed response. Potentially effective inhibitors are then tested in vivo in animal models of cancer to ascertain whether they may provide therapeutic benefit in terms of survival. Animal studies also provide valuable information regarding the toxicity and pharmacokinetic properties of the drug. Based on these preclinical studies, candidate molecules may be taken forward into the clinical setting. *KAT* histone lysine acetyltransferase, *KMT* histone lysine methyltransferase, *RMT* histone arginine methyltransferase, *PARP* poly ADP ribose polymerase (From M.A. Dawson and T. Kouzarides. Cancer epigenetics: From mechanism to therapy. *Cell* 150: 12–27, 2012. Reprinted with kind permission from Elsevier Limited)

to bear in mind the exact role of the DNMT inhibitors in cancer progression is not fully understood, and in some cases these drugs can elicit opposite response in different cancer cell lines. Recent data showed that treatment of MCF-7 breast cancer cells, which are largely epithelial, with 5-aza-2′-deoxycytidine may increase their ability for invasion and metastasis, concomitant with the upregulation of several pro-invasive genes. These observations raise concerns about the potential use of DNA methyltransferase inhibitors for the treatment of breast cancer [6].

HDAC inhibitors (HDACi) have been shown in preclinical studies to selectively target cancer cells with high specificity. The effects of HDACi include the induction of apoptosis and cell cycle arrest, and suppression of angiogenesis and tumor cell invasion. The anti-metastasis activity of HDACi is at least partially dependent on their potent capacity to upregulate E-cadherin expression. Butyrate, the first HDACi to be identified, induces cell cycle arrest and enhances cell-cell adhesion in two breast cancer cell lines, and these phenotypic changes can be inhibited by the addition of E-cadherin antibodies. Subsequently, butyrate was found to upregulate E-cadherin expression in colon cancer cells and in endometrial carcinoma cells.

As of today, there are at least 20 structurally different HDAC inhibitors in clinical trials, either in monotherapy or in combination therapy trials for hematological and solid tumors. HDAC inhibitors SAHA and romidepsin have been approved for the treatment of cutaneous T cell lymphoma [33, 34].

It is important to notice that the clinical effectiveness of the approved drugs is not necessarily dependent on their function to change the epigenome. Azacytidine is not a specific inhibitor of DNMTs, but a nucleoside analog that affects many cellular pathways. It is presently unclear to what degree the diverse and complex drug effects contribute to clinical responses to azacytidine. On the other hand, although SAHA is a highly specific HDAC inhibitor, the target enzymes are not specific for histones and include a wide range of non-histone proteins that are not involved in epigenetic regulation. Once again, clarifying the molecular mechanisms of epigenetic factors in cancer progression and designing more specific enzyme inhibitors are the key issues in the future application of epigenetic anti-cancer therapy.

4.7 Concluding Remarks and Future Perspective

Metastasis is life-threatening and accounts for 90 % of cancer mortality. Along the invasion-metastasis cascade, the initial commitment for carcinoma cells to move out is the EMT process. Extensive investigations have identified key signaling pathways and transcription factors that mediate EMT. Most of our current knowledge of metastasis epigenetics is essentially about the deregulated epigenome leading to abnormal expression of genes that induce or inhibit EMT. By contrast, while colonization in and adaptation to the distant foreign tissues is the final rate-limiting step for metastasis, our understanding to the molecular mechanisms of colonization is still limited, partly due to the difficulty to establish experimental models of this step. In fact, many patients may already have occult micrometastases at the time of primary cancer diagnosis; therefore targeting the final colonization step is a more appropriate therapeutic strategy to treat metastatic cancers. It is no doubt we should expect more studies to elucidate the molecular mechanisms governing metastatic colonization and the underlying epigenetic factors contribute to this process.

In recent years, we have witnessed the rapid development of many genome-wide based technologies. Global alteration of epigenomics is constantly observed in various types of invasive cancers. The integration of these data with the information coming from genomics and transcriptomics will exponentially expand understanding of cancer metastasis and yield better epigenetic biomarkers for detection, prognosis and therapy prediction. Considering that the effects of most of the epigenetic drugs are still nonspecific and may cause undesirable side effects, it will be necessary to design new agents against specific enzymes of the epigenetic machinery involved in specific types of cancer. At that time, epigenetics will truly enter the center stage of cancer research.

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Part II
Signaling Pathway and Cancer

Chapter 5
Regulatory Effects of Arsenic on Cellular Signaling Pathways: Biological Effects and Therapeutic Implications

Elspeth M. Beauchamp, Ruth Serrano, and Leonidas C. Platanias

Abstract Arsenic compounds exert important biological effects and arsenic trioxide has been approved by the Food and Drug Administration (FDA) for the treatment of patients with acute promyelocytic leukemia (APL). Much of arsenic's actions in cells reflect its ability to bind thiol groups in cellular proteins or to affect the production of reactive oxygen species (ROS), leading to the engagement and regulation of several cellular signaling pathways. Arsenic has been also shown to degrade abnormal fusion proteins found in myeloid leukemias. It has also been shown to effect NFκB, MAPK, mTOR and Hedgehog pathways which can modulate the viability of cancer cells. Many clinical trials have been performed to examine the clinical efficacy of arsenic trioxide alone or in combination with other agents in the treatment of various hematological malignancies. The continuous advances in basic and translational research and the better understanding of the mechanisms of action of arsenic should lead to more effective combinations with other agents that could result in better clinical outcomes.

Keywords Arsenic • Leukemia • Cancer • Cell signaling

5.1 Clinical Uses of Arsenic Trioxide

Arsenic has been used empirically for centuries, for the treatment of countless diseases, including syphilis, cancer, malaria, and ulcers [1]. It was first described as a drug to treat leukemia in 1878 [2]. In the modern medical era, one of the compounds of arsenic, arsenic trioxide, has shown significant clinical activity in certain malignant diseases, as discussed below.

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5.1.1 Acute Promyelocytic Leukemia (APL)

Over the last two decades there has been extensive evidence accumulated indicating that arsenic trioxide (ATO) has major clinical activity in the treatment of one form of acute myeloid leukemia (AML), acute promyelocytic leukemia (APL) and ATO was approved for the treatment of relapsed APL by the Food and Drug Administration (FDA) of the United States in 2001 [3]. This relatively rare variant of AML is associated with the reciprocal chromosomal translocation t(15;17) that brings together the promyelocytic leukemia (PML) gene on chromosome 15 and the retinoic acid receptor (RAR)α gene on chromosome 17 [4]. The resultant chimeric protein (PML–RARα) causes a maturation block of myeloid cells at the promyelocytic stage, resulting in the accumulation of abnormal promyelocytes in the bone marrow [4]. Historically, APL has been associated with a severe bleeding dysfunction associated with disseminated intravascular coagulation (DIC) and a fatal course of only weeks [5]. With the implementation of chemotherapy, a complete remission (CR) rate of 75–80 % in newly diagnosed patients was achieved, however the median duration of remission ranged from 11 to 25 months, with only 35–45 % of the patients being cured [4]. The introduction of a regimen consistent of *all-trans* retinoic acid (ATRA), which targets the RAR moiety of the fusion transcript, together with anthracycline-based chemotherapy dramatically raised the remission rate up to 90–95 % and the 5-year disease free survival (DFS) to 74 % [6]. Since the early 1990s, ATO was introduced for the treatment of relapsed APL, and has shown major clinical activity [7]. Since ATO is less toxic than chemotherapy, its role as a single agent in newly diagnosed patients is currently being researched with the aim to minimize the use of cytotoxic chemotherapy in this condition, especially for those with a compromised cardiac function and/or for older patients [8, 9].

5.1.2 Clinical Trials of ATO in Multiple Myeloma

In vitro studies have shown that ATO induces apoptosis in myeloma cells [10–13], therefore investigators have evaluated its potential in the treatment of refractory and relapsed multiple myeloma (MM) [14]. Some clinical activity was seen in a phase II study performed in 14 patients with refractory or relapsed MM [15]. In another trial using a higher but not as frequent dose of ATO, reduction of M-protein in serum of more than 25 % was obtained in eight patients (33 %), while six patients had stable disease, with a median duration response time of 130 days [16]. Investigators have also developed combination studies using ATO together other agents previously known to be useful for the treatment of this condition. Berenson et al. administered a combination of melphalan, ATO and ascorbic acid to 65 patients with MM who had failed more than two previous regimens [17]. This combination (also known as MAC regimen) produced objective responses in 31 patients (48 %), ranging from CR in two patients to minor responses in 14 of them [17]. More recently, the combination of MAC regimen plus bortezomib was evaluated in a different randomized trial and was found to be safe and well tolerated by patients [18]. Other combination

regimens including ATO have also demonstrated efficacy in patients with relapsed or refractory MM [19].

5.1.3 Myelodysplastic Syndromes

There has been also evidence for some clinical activity of ATO in the treatment of myelodysplastic syndromes (MDS). Hematologic improvement was obtained in MDS with the use of single agent ATO in two different trials [20, 21]. In other studies, thalidomide was used in combination with ATO in 28 patients with transfusion dependent MDS, accomplishing a response in 25 % of them, including one CR and responses in three of five patients with high baseline levels of EVI1, which is a known poor prognostic marker [22]. More recently, the combination of thalidomide, ATO, dexamethasone, and ascorbic acid (TADA regimen) was used in patients with myelodysplastic/myeloproliferative neoplasms (MDS/MPN) or primary myelofibrosis (PMF), achieving a response in 29 % of patients [23].

5.2 Effects of Arsenic on Cellular Signaling Pathways in Malignant Cells

5.2.1 Arsenic Compounds

Arsenic is found is two different oxidative states, As (III) or trivalent arsenic and As(V) or pentavalent arsenic. Pentavalent arsenic can substitute for phosphate and cause hydrolysis of compounds such as ATP [24]. Trivalent arsenic can bind to thiol groups in the cysteines of proteins in cells and alter their structure resulting in the modulation of protein stability, folding, and function, thus affecting cellular signaling pathways [24–26]. For instance, arsenic can bind to tubulin and other cytoskeletal proteins and affect polymerization and mitosis [27–30]. Arsenic can also affect signaling pathways through the production of reactive oxygen species (ROS) and there is evidence that it increases ROS in cells in two ways. First, arsenic can inhibit the activity of enzymes, such as thioredoxin reductase by its ability to bind via cysteine groups, which are involved in regulating the cellular redox state [31]. Second, methylation of arsenic during its cellular metabolism also leads to the production of ROS [32, 33].

5.2.2 Effects on Fusion Proteins in Leukemia

Arsenic trioxide has been shown to cause the degradation of multiple fusion proteins found in leukemia by various mechanisms. ATO's proposed mechanism of action in acute promeylocytic leukemia is via degradation of the PML-RAR fusion

protein [34]. In APL, the fusion protein alters the localization of PML from nuclear bodies, which contributes to aberrant cell growth [35, 36]. Arsenic trioxide targets both PML and PML-RAR to nuclear bodies in APL cells and leads to its subsequent degradation [37]. Targeting PML protein expression with arsenic has also been shown in quiescent leukemia initiating stem cells in CML [38]. A recent publication demonstrated that arsenic specifically binds to the PML zinc finger domain at cysteine residues displacing the zinc and causing a shift in secondary structure as well as aggregation that leads to increased sumolyation and degradation [39, 40]. Another recent publication showed that autophagy induction by ATO and ATRA also contributes to the degradation of the PML-RAR fusion protein [41].

Besides APL, arsenic has shown cytotoxicity in chronic myleogenous leukemia (CML), as well. It is of particular interest that historically, arsenic was used to treat CML in the nineteenth and twentieth centuries [1]. Imatinib combined with arsenic sulfide showed enhanced anti-leukemic effects over either agent alone in a mouse model of CML [42]. Recent evidence has shown that arsenic is cytotoxic in Ph + leukemia cells by degradation of the BCR-ABL fusion protein by the autophagic machinery, where p62 binds to BCR-ABL in the autophagosome [43]. Arsenic trioxide has been also shown to degrade another fusion protein, AML1/MDS1/EVI1, via targeting of the MDS1/EVI1 portion of the fusion protein [44]. The EVI1 portion contains two zinc finger DNA binding domains therefore similar to PML, arsenic could be binding to the cysteine residues in zinc finger domains in EVI1 and lead to the degradation of the fusion protein [40, 44].

5.2.3 mTOR Pathway

Arsenic has been shown to activate the mTOR pathway although the precise mechanism of such engagement is unknown (Fig. 5.1) [45]. Treatment with rapamycin or the dual PI3K/mTOR inhibitor, PI-103, was shown to enhance the antileukemic effects of arsenic, indicating that activation of mTOR occurs in a negative feedback manner in order to suppress the cytotoxic effects of arsenic [45, 46]. Therefore combining arsenic with mTOR pathway inhibitors could conceivably enhance its antileukemic effects in vivo and this needs to be examined in future work.

5.2.4 MAPK Pathways

Arsenic has been shown to affect the various MAPK pathways such as p38 MAPK, JNK and ERK. JNK activation has been shown to be important for the anti-leukemic effects of arsenic (Fig. 5.1) [47–49]. ATO-resistant APL cell lines showed little activation of JNK due to upregulation of glutathione (GSH) [47]. Treating cells with compounds that deplete GSH in cells enhance ATO's cytotoxic effects [47, 50]. Increased GSH levels in leukemia cells has been correlated with a decrease in

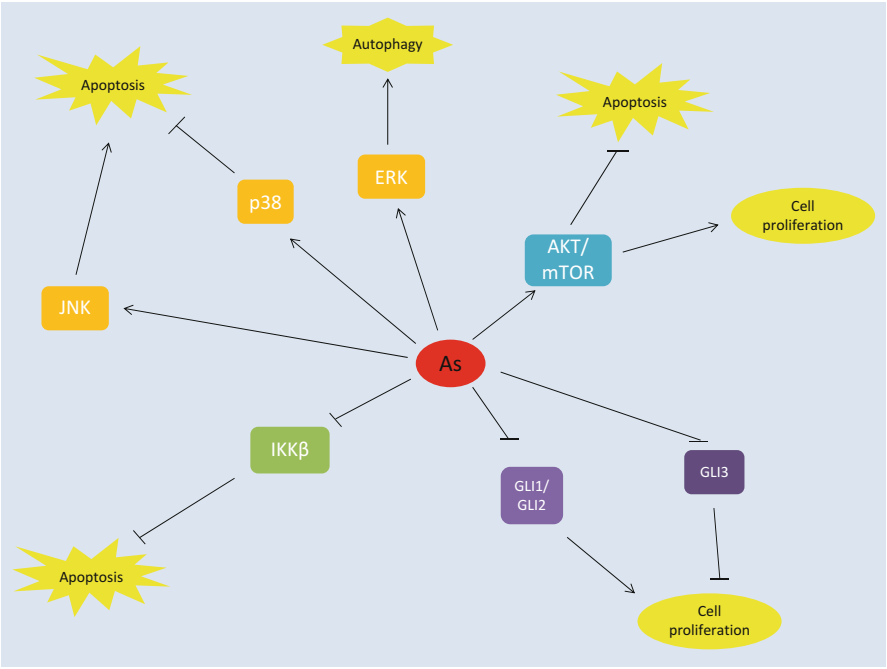

Fig. 5.1 Arsenic's positive and negative effects on cell viability and proliferation. Arsenic can affect MAPK pathways, by activating the MEK/ERK branch leading to the induction of autophagy. At the same time it can either activate p38 or JNK leading to the inhibition, or induction of apoptosis. Additionally, arsenic can activate the PI3K/mTOR pathway by activation of AKT signaling or mTOR signaling which leads to the inhibition of apoptosis and increase in cellular proliferation. Arsenic can inhibit GLI1 and GLI2 which leads to an inhibition of cellular proliferation. Arsenic's inhibition of GLI3, however, can lead to activation of cellular proliferation in some cellular contexts

sensitivity to arsenic, which could affect sensitivity by either GSH decreasing the amount of ROS in cells directly, or binding arsenic leading to its metabolism and subsequent excretion [51–53]. Ascorbic acid has been shown to synergize with arsenic in multiple myeloma and myeloid leukemia cells by decreasing GSH levels and increasing ROS levels [52, 54, 55]. In chronic lymphocytic leukemia (CLL), JNK activation was an early event leading to the upregulation of PTEN, which results in PI3K, AKT, NFκB inhibition, and an increase in ROS production [56]. In addition, combining arsenic with PI3K inhibitors was shown to enhance arsenic's cytotoxic effects on CLL cells [56].

Other studies have shown that arsenic modulates ERK activity. The induction of autophagy by arsenic trioxide was shown to be important for its antileukemic properties and the ERK pathway is required for induction of the autophagic state in this context [57]. ATO-dependent ERK2-mediated phosphorylation of PML has also been shown to lead to increased sumoylation/degradation of the PML protein and ultimately resulting in induction of apoptosis [58].

Arsenic trioxide also activates p38 MAPK in several leukemia cell types [59]. However, inhibition of p38 MAPK or its downstream effectors MNK or MSK1 attenuated the cytotoxic effects of ATO and/or increased JNK activation in leukemia cells [60–62]. This indicates that p38 MAPK is activated as a negative feedback loop in leukemia cells, which limits arsenic's cytotoxicity. Co-treatment of breast cancer or leukemia cells with ATO and MEK inhibitors leads to a greater induction of apoptosis, suggesting a possible therapeutic approach to enhance arsenic's cytotoxic effects [63, 64].

5.2.5 Effects on the NFKB Pathway

The canonical NFκB pathway has been shown to be inhibited by arsenic. When the canonical NFκB pathway is not active, the negative regulator IκB binds to the NFκB dimer and prevents it from translocating to the nucleus [65]. Activation of this pathway in response to TNFα or other stimuli leads to activation of the IKK complex [65]. IKK phosphorylates IκB leading to its degradation, which allows NFκB to translocate to the nucleus and activate pro-tumorigenic genes that help lead to the evasion of apoptosis [66]. In multiple myeloma cells, arsenic trioxide was shown to prevent NFκB activation by TNFα [10]. Arsenic can directly bind to IKKβ at cysteine residue 179 in the activation loop of the catalytic subunit of IKKβ and inhibit its activity, to engage the NFκB canonical signaling (Fig. 5.1) [67]. Since IKKβ can have effects independently of NFκB such as by regulating MAPK and mTOR pathways [66], the inhibition of IKKβ by arsenic can also conceivably effect those pathways in addition to NFκB.

5.2.6 Hedgehog Pathway

Recent work has shown that arsenic can inhibit the hedgehog pathway by inhibiting GLI1/2 (Fig. 5.1) [68, 69]. Such inhibition was shown to be at the level of GLI1/2 because ATO was found to inhibit hedgehog signaling when GLI1/2 was overexpressed or in $SUFU^{-/-}$ MEFs, in contrast to upstream pathway inhibitors that cannot inhibit Hh signaling in this context [68, 69]. Notably, some tumors activate the pathway by overexpression of ligand, patched inactivation or mutations that activate Smoothened [70–76]. Other cancers, however, can activate the pathway at the level of GLI, independent of Smoothened or Patched, either by mutations in negative regulators SUFU or REN, chromosomal amplification of GLI, chromosomal translocations that involve GLI, an increase in GLI protein stability or activation via non-canonical mechanisms involving other pathways [77–89]. Arsenic is able to inhibit the growth of both upstream activated medulloblastoma cancer cell lines as well as Ewing sarcoma cells lines which have activation of GLI1 independently of SMO [68].

The exact mechanisms by which arsenic inhibits GLI1/2 still need further investigation. Since one of the studies demonstrated that arsenic can directly bind to GLI1 [68] and given prior evidence of arsenic's ability to bind to cysteines in the zinc finger domain of PML, it is highly plausible that ATO binds to the zinc finger domains in GLI1. However, this remains to be directly addressed in future studies and the overall mechanisms by which arsenic affects GLI function necessitates further investigation.

Another study showed that arsenic activates Hedgehog signaling [90]. The authors of that study found that arsenic activated GLI1/Hedgehog signaling in these cells by inhibiting the GLI3 repressor. However, in this study sodium arsenite was used, whereas the other two studies used arsenic trioxide. It is possible that sodium arsenite has preferential binding to GLI3 over GLI1 and GLI2 and thus activates signaling instead of repressing it. Notably, sodium arsenite has been previously shown to have opposing effects to the ones of arsenic trioxide in other cancer models. For instance, arsenic trioxide promotes apoptosis in breast cancer cell lines [91, 92], while sodium arsenite binds to the estrogen receptor-α (ER-α) and increases the proliferation of MCF-7 cells [93]. Arsenic trioxide and sodium arsenite have been also shown to exhibit differential effects when combined with radiation [94].

The precise mechanisms of how arsenic induces autophagy are not known, other than the requirement for MEK/ERK signaling [54]. Recent evidence suggests that the hedgehog pathway antagonizes autophagy through inhibition of autophagosome synthesis most likely through repression of genes required for autophagy [95]. Thus, the inhibition of the hedgehog pathway by arsenic could mechanistically contribute to its ability to induce autophagy and this hypothesis remains to be examined in future studies.

5.2.7 Effects on Nuclear Receptor Pathways

Arsenic has been shown to alter multiple nuclear receptor pathways. Notably, it has been shown to directly bind and inhibit the glucocorticoid receptor [96]. Nuclear receptor function has been shown to be inhibited by arsenic trioxide though JNK activation and phosphorylation of the retinoid X receptor (RXR) [97]. Arsenic's effects on the estrogen receptor are controversial as multiple groups have shown differential effects. As mentioned previously, sodium arsenite can bind to the ligand pocket of ER-α and activate it, leading to proliferation of MCF-7 cells [93]. Arsenic trioxide was shown to lead to a decrease in expression of ER-α in ER-positive breast cancer cell lines, resulting in suppression of cellular proliferation [98, 99]. More recently arsenic trioxide treatment was found to result in increased expression of ER-α in ER-negative breast cancer cells by promoting demethylation of the promoter, leading to re-sensitization to endocrine therapy [100, 101]. The differences in effects may be due to differences in cell contexts (ER-positive vs. ER-negative cells) as well as mentioned previously the differential effects of sodium arsenite and arsenic trioxide.

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Chapter 6
Nuclear Factors Linking Cancer and Inflammation

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Abstract An association between inflammation and cancer has long-been known, but the past decade has witnessed a spurt in the research linking the two processes. On the one hand chronic inflammation predisposes to cancer, on the other, neoplastic transformation predisposes towards an intrinsic pro-inflammatory microenvironment, which further promotes the progression of the malignancy. Irrespective of the stimulus, whether extrinsic (bacteria, viruses, non-healing wounds, irritants etc.) or intrinsic (oncogenes, protein kinases etc.), all signals that trigger the inflammatory microenvironment in tumor cells converge in the nucleus and coordinate inflammatory transcriptional activity by activating various nuclear/transcription factors. This forms a vicious cycle, further promoting inflammation, facilitating tumor progression, proliferation, survival and angiogenesis. This chapter focuses on transcriptional mediators intrinsic to tumour cells that enhance the inflammatory processes.

The well known nuclear/transcription factors important for this linkage are NFkB, HIF1α, AP-1 and STAT-3. The cross talk between these factors results in a complex web of signalling processes that have a marked influence on the cellular phenotype, cell-cell interactions and the interaction of the tumour with the microenvironment. This chapter focuses on the above mentioned factors and their effects, while also looking at the possibilities of utilising the same for therapeutic intervention.

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Abbreviations

AOM	Azoxymethane
AP-1	Activator protein 1
ARP 2/3	Actin-related protein 2/3 complex subunit 1B
ATF	Activating transcription factor
BAK	BCL2-antagonist/killer
BAX	BCL2-associated X protein
Bcl-2	B-cell CLL/lymphoma *2*
Bcl-xl	B-cell lymphoma-extra large
CapG	Capping protein (actin filament)
cIAP2	Baculoviral IAP repeat-containing protein 3
c-Rel	Proto-oncogene *c-Rel*
COX-2	Cyclooxygenase-2
CRE	cAMP response element
CXCL	Chemokine (C-X-C motif) ligand
DEN	Diethylnitrosamine
DSS	Dextran-sulphate sodium
EGF	Epidermal growth factor
EGFR	Epidermal growth factor
EMT	Epithelial-Mesenchymal Transition
ERK	Extracellular signal-regulated kinase
FIH	Factor inhibiting HIF
Fos	FBJ murine osteosarcoma viral oncogene homolog
Fra	Fos–gene family member
GBM	Glioblastoma multifome
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HIF1α	Hypoxia-inducible factor 1 α
IFN-γ	Interferon-gamma
IGSF4	Immunoglobulin (Ig)-like intercellular adhesion molecule
IκB	Inhibitor of kappa light polypeptide gene enhancer in B-cells
IKK	IκB kinase
IL-1β	Interleukin 1-beta
Inos	Inducible Nitric acid synthase
JNK	c-Jun N-terminal kinase
JUN	*jun* proto-oncogene
KC	Keratinocyte chemokine
LPS	Bacterial lipopolysaccharides
MAF	Musculoaponeurotic fibrosarcoma
MAPK	Mitogen-activated protein kinase

Mcl-1	Myeloid cell leukemia sequence 1
MIP	Major intrinsic protein of lens fiber
MMP	Matrix metalloproteinase
MUC1	Mucin 1, cell surface associated
MYC	v-*myc* myelocytomatosis viral oncogene homolog
NEMO	NF-kappa B essential modulator also known as IKKγ
NF-Kb	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIK	NF-κB inducing kinase
NSAID	Nonsteroidal anti-inflammatory drug
PDCD4	Programmed cell death protein 4
PHDs	Proline hydroxylases
PIM-1	Proto-oncogene serine/threonine-protein kinase
PTEN	Phosphatase and tensin homolog
RAF1	v-*raf*-1 murine leukemia viral oncogene homolog
RAS	*RAS* oncogene
RelA	v-rel reticuloendotheliosis viral oncogene homolog A
RelB	v-rel reticuloendotheliosis viral oncogene homolog B
RHD	Rel homology domain
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SPARC	Secreted protein acidic and rich in cysteines
STAT3	Signal transducer and activator of transcription 3
STAT6	Signal transducer and activator of transcription 6
TAMs	Tumor associated macrophages
TGF-β	Transforming growth factor beta
TNF	Tumor necrosis factor
TPA	12-0-tetradecanoylphorbol 13-acetate
TRAMP	Transgenic adenocarcinoma of mouse prostrate
TRE	TPA response element
TWIST	Twist homolog (Drosophila)
VEGF	Vascular endothelial growth factor
VHL	Von Hipple Lindau
ZEB	Zinc finger E-box binding homeobox

6.1 Introduction

Inflammation has long been known as a protective measure to tissue from injury, infection or irritation. While acute inflammation occurs as a part of immediate defense mechanism, chronic inflammation due to persistent infections can lead to several diseases including arthritis, cardiovascular, neurological disorders, cancer etc. [1].

Association between inflammation and cancer has long been known. Initial information linking inflammation with carcinogenesis dates back to 1863 when Rudolf Virchow noted malignant neoplasm arise at regions of chronic inflammation

and reasoned that various "irritants" cause tissue injury, inflammation and increased cell proliferation [2, 3]. Studies way back in early 1960s have pointed out the role of inflammation in the promotion of established cancers [4]. In the recent years, it has been proven that the inflammatory microenvironment driven by the presence of cytokines in solid tumors have major role in tumor progression and invasion [5, 6].

Epidemiological studies have shown that around 1.2 million cases of infection related malignancies per year are caused due to chronic inflammation as a result of bacterial and viral infections [6]. Viruses such as human papillomavirus, hepatitis C virus and hepatitis B virus, in addition to inhibiting tumor suppressor proteins or promoting tumor promoter proteins, can cause malignancy through inflammation related mechanisms [7–9]. Along with gastrointestinal tract, other organs with high susceptibility to tumor development following a chronic inflammation are the lungs, bladder, liver, pancreas and oesophagus etc. [3].

Inflammation is an indispensable participant in neoplastic progression [6, 7] and suppression of pro-inflammatory pathways demonstrates potential for the prevention and treatment of cancer [10]. The importance of inflammation in tumor initiation and malignant progression has become one of the major focus and researchers are analyzing the mechanism linking the two.

The trigger(s) for inflammatory microenvironment in tumors can be extrinsic for example by inflammatory and infectious conditions [6, 8] or intrinsic stimulus through oncogenes and cytokines [5, 11]. Persistence of chronic inflammation is one of the major extrinsic factors involved in neoplastic transformation of the cells and higher risk of developing many age related malignancies [12]. A lot of evidence has established that inflammation regulates different stages of tumor development, such as initiation, promotion, invasion and metastasis [5], whereby various pro-inflammatory mediators triggered by inflammation aid tumor progression by regulating cascades of cytokines, chemokines, adhesion and pro-angiogenic activities [6].

In addition, intrinsic factors like oncogenes triggering the pro-cancer inflammatory microenvironment in solid tumors are equally important. The role of protein tyrosine kinase cascade [13–15] was the first evidence on the role of intracellular molecule in triggering inflammatory program. Similarly, RAS-RAF signaling pathway [16–18], MYC [19], TGFβ etc. are known to promote inflammatory microenvironment by upregulating the expression of various cytokines and inflammatory modulators like COX-2 etc.

Irrespective of the stimulus, whether extrinsic or intrinsic, all signals that trigger the inflammatory microenvironment in the tumor cells converge in nucleus and coordinate inflammatory transcriptional activities by activating various nuclear/transcription factors [20]. It forms a vicious cycle further promoting the inflammatory microenvironment of the tumor cells facilitating tumor progression, proliferation, survival and angiogenesis.

The well known nuclear/transcription factors activated by these proinflammatory signaling pathways, upregulating the inflammatory microenvironment and cancer progression are NFkB, HIF1α, AP-1 and STAT-3. This chapter focuses on the role of these nuclear factors in triggering inflammatory microenvironment in cancers, helping tumor cells to survive, proliferate and promote tumor aggressiveness.

6.2 NF-κB

6.2.1 Introduction

NF-κB (nuclear factor kappa light chain enhancer of activated B cells) is a protein complex that controls transcription of a number of genes involved in inflammation, cell proliferation and immune response. Five members of NF-κB family have been identified in mammals: RelA/p65, c-Rel, RelB, NF-κB1/p50 and NF-κB2/p52. All proteins of the NF-κB share a Rel homology domain(RHD) in their N-terminus, which is required for DNA binding, homo and heterodimerization, nuclear localization and inhibitor (IκB) binding [9]. Only RelA, Rel B and c-Rel contain Transactivation Domains (TDs- required to act as transcriptional activators) at their C-terminal regions. p50 and p52,which do not possess these TDs, are produced as p105 and p100 precursors respectively. p105 and p100 are then processed by ubiquitin proteosome pathway that cleaves the C-terminal ankyrin repeats(IκB like portion) containing region to respectively generate p50 and p52 [21].

Two distinct pathways lead to activation of different NF-kB transcription factors (Fig. 6.1). The classical or the cannonical pathway triggered by pro-inflammatory cytokines in bacterial and viral infections led to activation of the IκB kinase (IKK). IKK complex, which has two catalytic subunits, IKK- α and IKK-β and a regulatory subunit, IKK-γ (NEMO), phosphorylates NFκB-bound IκBs leading to release of

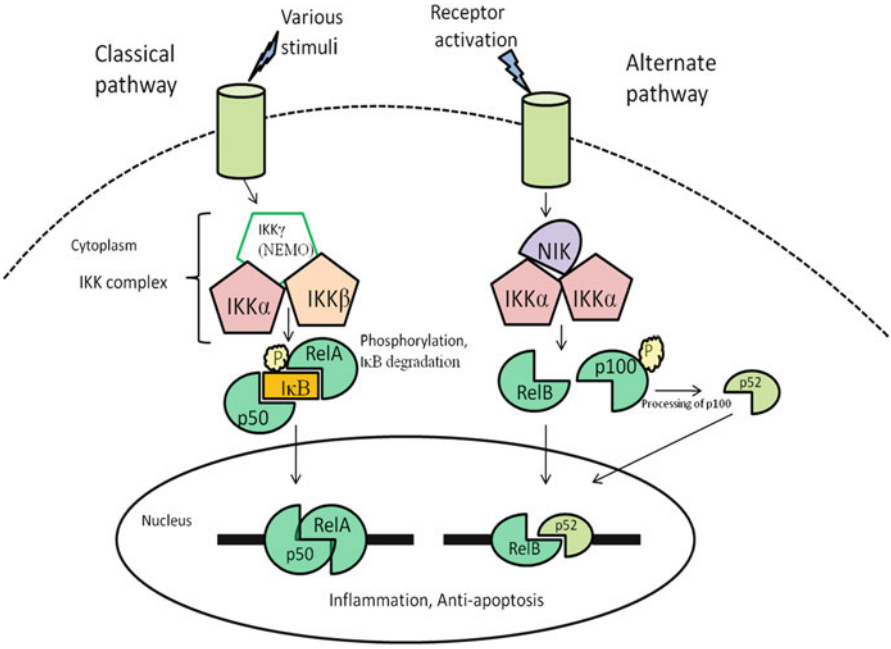

Fig. 6.1 NF-κB signaling via classical and alternative pathways

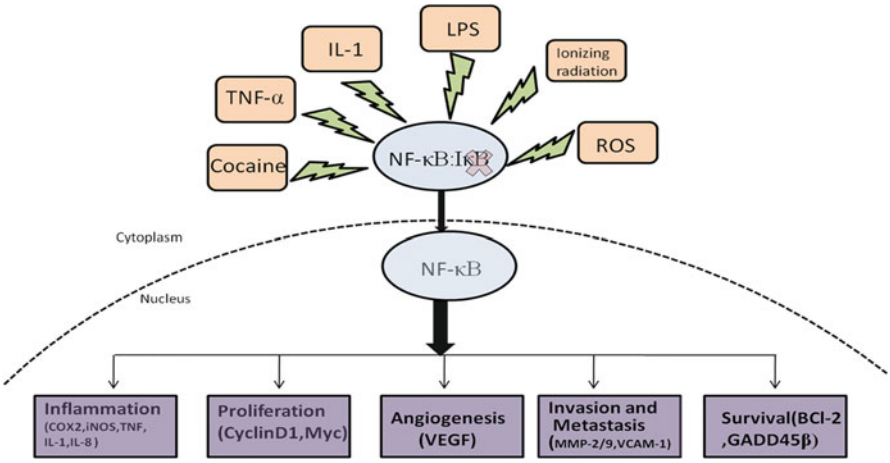

Fig. 6.2 NF-κB activation and its functional effects on cells

NF-κB. The free NF-κB is the activated form which then translocates to the nucleus where it can 'turn on' the expression of specific genes that have DNA-binding sites for NF-κB. The phosphorylated IκB undergoes proteosomal degradation [21].

The non-cannonical pathway involves activation of NF-κB inducing kinase (NIK) in response to certain members of TNF family, which activates IKK-α (independent of IKK-β and IKK- γ) leading to phosphorylation and processing of p100 to p52 [22]. Both the pathways mediate different immune responses by activating different sets of genes.

6.2.2 NF-κB Activation and Inflammatory Networking in Cancer

A wide variety of inflammatory stimuli (e.g. infectious agents, cytokines and carcinogens) are known to trigger NF-κB [23]. Known inducers of NF-κB include reactive oxygen species(ROS), tumor necrosis factor (TNFα), interleukin 1-beta (IL-1β), bacterial lipopolysaccharides (LPS), ionizing radiation and cocaine (Fig. 6.2) [1, 24, 25].

Aberrant NF-κB activation leads to induction of a diverse array of genes involved in tumorogenesis. These include the expression of anti-apoptotic genes promoting cancer cell survival, the expression of cyclins and proto-oncogenes leading to cell proliferation, the expression of matrix metalloproteinases and cell adhesion genes promoting metastasis and induction of genes responsible for new blood vessel growth stimulating angiogenesis (Table 6.1) [26]. All these actions are also very important in defense mechanisms of the body towards infection and injury but in a tumor microenvironment, these inappropriately regulated set of genes may provide an advantage to the malignant cells to proliferate and survive.

Table 6.1 NF-κB target genes involved in oncogenesis

Gene	Function	References
Bcl-2	Pro-survival factor	Catz and Johnson 2001
IL-6	Interleukin-6, inflammatory cytokine	Son et al. 2008
IL-8	Interleukin-8, alpha-chemokine	Kang et al. 2007
ICAM-1	Intercellular adhesion molecule-1	Bunting et al. 2007
Epidermal Growth Factor Receptor	Receptor for EGF	Thornburg and Raab-Traub 2007
Bax	Pro-apoptotic Bcl-2 homologue	Grimm et al. 2004
Fas-Ligand	Inducer of apoptosis	Singh et al. 2006
TRAF-1	TNF-receptor associated factor	Schwenzer et al. 1999
Angiopoietin	Tie-2 receptor ligand	Scott et al. 2005
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor	Bunting et al. 2007
EPO	Erythropoietin	Figueroa et al. 2002
A20	TNF-inducible zinc finger	Krikos et al. 1992
ABIN-3	NF-kB inhibitor	Verstrepen et al. 2007
E2F3a	Cell cycle regulator	Cheng et al. 2003
HIF-1alpha	Hypoxia-inducible factor	Bonello et al. 2007
MMP-3, matrix metalloproteinaase-3	Secreted collagenase involved in metastasis	Borghaei et al. 2004
MMP-9, matrix metalloproteinaase-9	Secreted collagenase involved in metastasis	Yan et al. 2004
Cyclin D1	Cell-cycle regulation	Toualbi-Abed et al. 2008
Cyclin D2	Cell-cycle regulation	Iwanga et al. 2008
Gadd45beta	DNA repair/cell cycle	Qiu et al. 2004

6.2.3 NF-κB: A Defensive Route to Malignancy

Since its discovery, many studies have shown NF-κB to be a key regulator of inflammation [27]. Indeed, promoters of many genes encoding for cytokines and chemokines contain NF-κB binding sites [28]. The first hint implicating an association between NF-κB and cancer was the observation that it shares sequence homology with viral oncoprotein v-Rel [29]. This, together with the findings of activated NF-κB in cancers hypothesized a link between cancer and inflammation. Recently many studies using mice models have been carried out establishing a central role of NF-κB in cancer. NF-κB is found to have role in all three stages of carcinogenesis (Tumor initiation, tumor promotion and tumor progression) [30].

6.2.3.1 NF-κB–A Tumor Initiator

Many studies have established that infection with *Helicobacter pyroli* in gastric epithelium leads to NF-κB activation [30]. The first molecular evidence linking inflammation to tumor initiation was shown by Houghton et al. in 2004, in which a mouse model of Helicobacter induced gastric cancer was used. It was shown that chronic

inflammation caused by bacterial inflammation can lead to gastric mucosa erosion followed by repopulation of bone marrow derived cells at the site of erosion. These repopulated cell differentiated into gastric epithelial phenotype giving rise to gastric carcinoma [31]. NF-κB is also involved in the induction of iNOS (inducible Nitric acid synthase). iNOS has been shown to be involved in cellular changes like transformation of normal cells, triggering angiogenesis and metastasis of malignant cells [32].

6.2.3.2 NF-κB–A Tumor Promoter

Recent work in mouse studies has provided strong genetic evidence demonstrating a crucial role of NF-κB in tumor promotion. Studies on Mdr2 knockout mice have found spontaneous development of hepatocellular carcinoma (HCC) within 8–10 months of birth due to inflammation of the bile duct followed by cholangitis and cancer [5, 33]. TNF-α, upregulated in the endothelial and inflammatory cell had led to activation of NF-κB in the adjacent hepatocytes. Suppressing NF-κB through expression of a non-degradable IκBα variant (IκB super-repressor) or by using neutralizing antibodies for TNF-α resulted in apoptosis of premalignant hepatocytes and failure to progress to hepatocellular carcinoma [33]. Activation of NF-κB through TLR signaling was found in mouse model of colitis-associated cancer on exposure to mutagen azoxymethane (AOM) followed by administration of dextran-sulphate sodium (DSS) salt led to development of cancer. Deletion of IKK-β in the intestinal epithelial cells resulted in decrease in tumor incidence and an increase in expression levels of pro-apoptotic BAX and BAK proteins indicating a role of NF-κB in suppressing apotosis in colon cancer [5].

6.2.3.3 NF-κB: Role in Tumor Progression

Once a tumor is established, the role of NF-κB does not end and it continues to be a central signaling molecule in tumor progression. NF-κB is known to regulate many genes which are involved in tumor invasion and metastasis such as serine proteases, adhesion molecules, MMPs, and chemokines [6, 34]. Also, NF-κB induces iNOS leading to increased production of reactive nitrogen species (RNS) which might result in generation and accumulation of additional DNA mutations that drive tumor progression [32]. In addition to inducing the expression of inflammatory cytokines, NF-κB is also seen in polarization of tumor associated macrophages or TAMs [35]. In response to different stimuli, macrophages undergo different activation programmes. M1 macrophages (which produce IL-12, GM-CSF and TNF when exposed to IFN-γ) are proposed to be tumoricidal, whereas M2 macrophages (producing IL-10, VEGF, iNOS when exposed to IL-4) are proposed to be tumor promoting [5]. It was shown that inhibition of NF-κB in macrophages converts them from M2 tumor promoting phenotype to M1 cytotoxic phenotype, resulting in tumor regression [36].

NF-κB pathway is extensively studied in both solid tumors and hematological malignancies (Fig. 6.3). Constitutive activation of NF-κB is seen in GBM tumors and inhibition of NF-κB activity induces apoptosis in glioma [37]. Deletion of

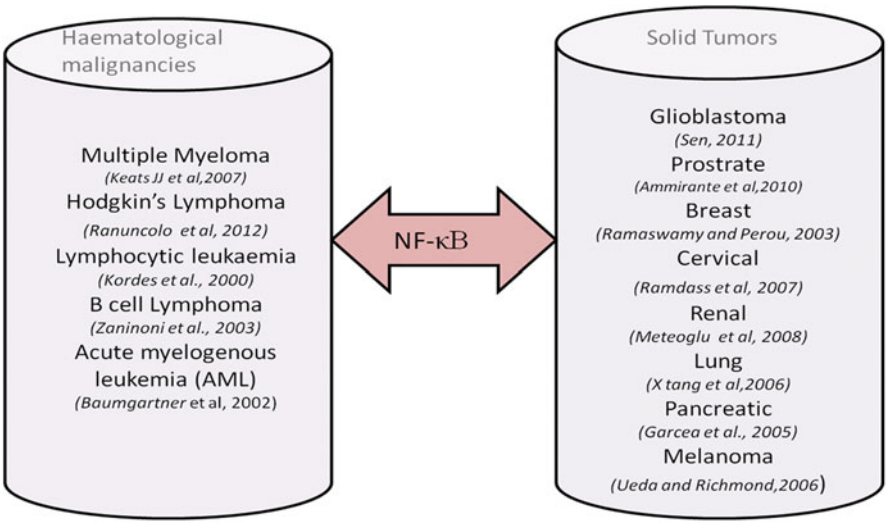

Fig. 6.3 Human cancers (heamatological and solid) with activated NF-κB expression

NFKBIA, a gene coding for IκBα is a frequent oncogenic mutation seen in glioblastoma tumors and is mutually exclusive with EGFR amplification. It was shown that deletion of NFKBIA (which normally inhibits EGFR signaling), and thus activation of NF-κB, had an effect that was similar to the effect of EGFR amplification in the pathogenesis of glioblastoma and was associated with comparatively short survival [38]. NF-κB is found to be activated in castration resistant prostate cancer [29]. Using a TRAMP (Transgenic adenocarcinoma of mouse prostrate) model, it was shown that deletion of IKKβ in epithelial cells did not have any effect on tumor development and progression [39]. Furthermore, IKKα and not IKKβ enhanced metastatic progression in these mice [40]. Constitutive NF-κB activation has been shown in primary myeloma cells and cell lines. Furthermore, inhibitors of NF-κB signaling such as proteosome inhibitors, IKK inhibitors and IκB phosphorylation inhibitors have induced apoptosis and inhibited growth of multiple myeloma cells [29]. Most of the genetic alterations in multiple myeloma activate both classical and alternate pathways. These studies also showed stabilization of NIK in multiple myeloma (which is normally degraded in normal cells) which is required to activate both the pathways, thus leading to B cell survival [41].

6.2.4 NF-κB and Its Role in Cancer Stem Cells

The connection between NF-κβ in cancer stem cells has been well explored in breast cancers. A membrane bound receptor tyrosine kinase Her2, which controls NF-κB through the canonical pathway is known to be overexpressed in 30 % of breast cancers. It has also been shown to play a role in regulating cancer stem cell population [42]

In a mouse model of Her2 breast cancer, NF-κB suppression (using IκB super repressor) in mammary gland was seen to decrease CD44-positive cells. Also, there was reduced formation of non-adherent mammospheres in cell lines derived from Her2-dependent tumors in which NF-κB was inhibited. This phenomenon was attributed to reduced expression of stem cell regulators SOX-2 and Nanog as a result of NF-κB inhibition [43].

6.2.5 Anti-tumorigenic Effects of NF-κB

Although NF-κB is known to promote cell survival and transcriptionally activate tumor promoting cytokines in inflammatory cells, its activation in epithelial cells can have either positive or negative effect on tumor development. NF-κB inhibition has been shown to increase carcinogenesis in mice. In DEN (diethylnitrosamine -a pro-carcinogen) induced hepatocarcinoma mouse model, deleting IKK-β in hepatocytes resulted in increase in tumor growth and size [44]. In skin cancer mouse model, tumor was initiated by administration of 7,12-dimethylbenzanthracene (DMBA-a carcinogen) followed by phorbol esters administration. Inhibition of NF-κB in the epidermis in this model promoted development of squamous cell carcinoma [45, 46]. TNF-deficient and TNFR1-deficient mice were, however, found to be resistant [47].

6.2.6 NF-κB: A Target in Therapeutics

The findings that NF-κB is seen to be activated in many malignancies provide a strong rationale for the use of NF-κB inhibitors as therapeutics in treating cancer. Efforts have been made by the pharmaceutical companies to develop IKKβ and NF-κB inhibitors [48]. Such an inhibitor, should specifically prevent NF-κB activation in malignant cells without showing any side effects on normal cells. Also, the dose should be such that so as to minimize systemic toxicity and avoid broad suppression of innate immunity. Many reports have shown the role of these NF-κB/IKKβ inhibitors as anti-tumor compounds in experimental cancer models [49, 50]. However, such a drug is unlikely to be effective alone in tumor regression and can be used as adjuvants for existing chemotherapeutic drugs or radiation therapy. However, excessive and prolonged NF-κB inhibition can be detrimental owing to its important role in innate immunity. Due to these factors, NF-κB inhibitors have not been clinically approved till date.

Also, selective interference in upstream activation or downstream targets of NF-κB such as, TNF-α, IL-6 or targeting individual cytokines can also prove effective [30]. Targeting proteosome by using inhibitors that can block NF-κB activation can also be developed as a good therapeutic strategy, as is the case with bortezomib, which has reached phase III clinical trial in treating multiple myeloma [51, 52].

Curcumin, also has been perpetually a promising molecule for NF-κB inhibition. It has been shown to inhibit NF-κB activation and cell proliferation in many cancers

[51, 53]. Curcumin blocks the IKK β mediated phosphorylation and degradation of IκBα, thus NF-κB remains bound to IκBα in the cytoplasm and is not able to enter the nucleus to activate transcription [53]. Recently, curcumin has been tested for phase II clinical trial against pancreatic cancer in which curcumin downregulated NF-κB expression [54].

6.3 HIF-1α: A Synergistic Link Between Cancer and Inflammation

Solid tumors are characterized by intratumoral hypoxia that result from dysregulated cell proliferation. Physiological responses triggered by hypoxia impact on all critical aspects of cancer progression, including immortalization, transformation, differentiation, genetic instability, angiogenesis, metabolic adaptation, autocrine growth factor signaling, invasion, metastasis, and resistance to therapy [55].

Hypoxic conditions in in-vitro as well as in-vivo result in induction of HIF-1α (Hypoxia Inducible Factor), whereas the beta subunit is not sensitive to oxygen. Oxygen sensitivity of HIF-1α is conferred by a family of oxygen-dependent hydroxylase enzymes including three proline hydroxylases (PHDs) and one asparigine hydroxylase known as the factor inhibiting HIF (FIH). These hydroxylases promote oxygen-dependent hydroxylation of HIF-1α leading to its ubiquitylation by the Von Hipple Lindau (VHL) E3 ligase and subsequent degradation in the proteasome. This effect is reversed in hypoxia leading to HIF-1α stabilization and activation [56].

HIF-1α, on activation is known to stimulate the transcription of a number of genes linking inflammation and tumor survival such as vascular endothelial growth factor (VEGF), Carbonic anhydrase IX, iNOS, COX-2 and several glycolytic enzymes. HIF-1α can also be activated under normoxia in response to pro-inflammatory cytokines IL-1β and TNF-α. The IL-1β induced HIF-1α activity is mediated by NF-κB [37].

In innate immunity, HIF-1α is shown to regulate phagocyte immune functions by inhibiting apoptosis, releasing antimicrobial peptides, inducing iNOS and promoting the expression of NF-κB regulated cytokines [56]. HIF-1α is also shown to mediate NF-κB activation in neutrophils under anoxic conditions [57, 58]. Hypoxia is also hypothesized to down regulate adaptive immune response by inhibiting IFN-γ production and T cell activation [57]. HIF-1α, therefore promotes tumorigenesis by curtailing adaptive immune system and enhancing innate immune system (which during chronic inflammation can lead to cancer).

6.3.1 HIF1α -Hypoxia- NF-κB: Who Regulates Whom?

Studies linking NF-κB signaling pathway to hypoxia dates back to 1994 when it was shown by Koong et al. (1994) that exposure of cells to hypoxia (0.02 % O2)

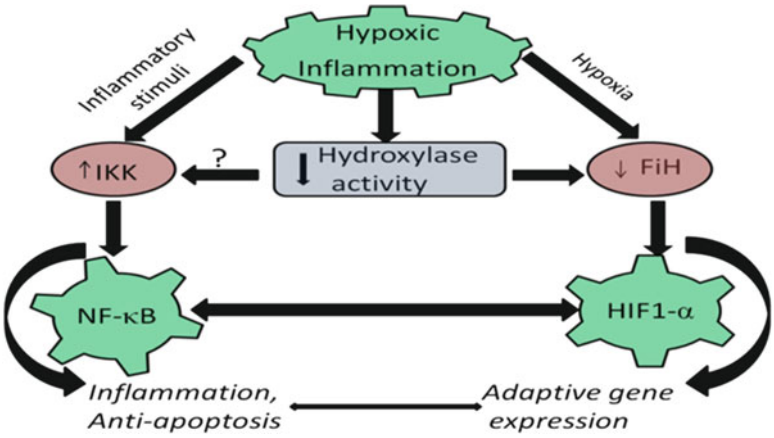

Fig. 6.4 Crosstalk between NF-κB and HIF1 in hypoxic inflammation

resulted in IκBα degradation and increased NF-κB DNA binding activity . This link between hypoxia and NF-κB was then supported by several reports demonstrating activation of NF-κB dependent genes such as TNF-α , IL-6 and COX-2 in response to hypoxia [59]. Furthermore, it has been proved recently, that hypoxia activates NF-κB through a pathway involving activation of IKKβ, which in turn phosphorylates IκBα, liberating it from NF-κB (Fig. 6.4) [60].

The presence of conserved motif in activation loop of IKKβ and IKKα for hydroxylation by prolyl hydroxylase (PHD-which also inhibits HIF-1α activation) has been identified. Site directed mutagenesis of the proline residue in these putative IKKβ hydroxylation site did not induce IKKβ in hypoxia. Moreover, it has been shown that knockdown of PHD in HeLa cells led to activation of NF-κB. These findings hypothesize that hypoxia releases NF-κB repression through decreased PHD-dependent hydroxylation of IKKβ, in a manner, similar to that of HIF-1α activation in hypoxia [61].

6.3.2 HIF-1α: NF-κB Crosstalk

A plethora of evidence now exists that hypoxia activates both HIF-1α and NF-κB, which act as transcriptional activators for a number of genes involved in tumor development [56, 59, 61]. However, recent studies have also exhibited an interdependent role of HIF-1 and NF-κB in regulating the expression of each other. HIF-1α, apart from being induced by hypoxia, is also shown to be activated in response to a number of non-hypoxic stimuli including bacterial LPS, TNF-α, ROS and IL-18 in NF-κB dependent manner by increasing HIF-1α mRNA levels [62]. It was shown that expression of the NF-κB subunits p50 and p65 enhances HIF-1α mRNA levels,

whereas blocking NF-κB by an inhibitor attenuated HIF-1α mRNA induction by hypoxia [63]. Furthermore, presence of an NF-κB binding site at position within the HIF-1α promoter has been identified and mutation of this site abolished induction by hypoxia [64].

Interestingly, mice lacking IKKβ in different cell types resulted in defective induction of various HIF-1α target genes including VEGF [[60]. The role of HIF-1α in regulating NF-κB signaling has also been reported. Mice overexpressing HIF-1α in keratinocytes showed increased NF-κB activity and increased expression of pro-inflammatory targets including macrophage inflammatory protein-2 (MIP-2/CXCL2/3), keratinocyte chemokine (KC/CXCL1), and TNF-α, leading to hyper-responsiveness to inflammatory stimuli. HIF-1α -induced NF-κB activation was due to phosphorylation of IκB (leading to its degradation) and Ser276 on p65, thus enhancing its nuclear localization and transcriptional activity [65]. Therefore, there is an interdependency between HIF-1α and NF-κB signaling leading to their synergistic role in tumor development and progression.

6.4 AP-1

6.4.1 Introduction

AP-1 (**Activator Protein 1**), one of the most extensively studied transcription factors, is involved in regulating the expression of a variety of genes by forming either a homodimer or heterodimers . The dimers are formed between the proteins belonging to different families like Jun, Fos, Fra, MAF (musculoaponeurotic fibrosarcoma) and ATF (activating transcription factor) [66–69]. There are multiple proteins included in each protein family of AP1 complex, like in Jun family, there are c-Jun, JunB and JunD; in Fos family- c-Fos and FosB; in Fra family - Fra1 and Fra2; in ATF family - ATFa, ATF2 and ATF3; and in Maf family - v-Maf, c-Maf, Nrl, MafB, MafF, MafG and MafK [67, 70–73].

The Jun and ATF family of proteins form homodimers and Jun also forms heterodimers with Fos, ATF and Maf [66, 68, 69]. The Fos proteins forms stable heterodimers with Jun but cannot form a stable homodimer [68, 69]. The cMaf and Nrl form heterodimers with Jun and Fos but other Maf proteins like MafB, MafF, MafG and MafK forms heterodimers only with Fos [74].

Among Jun proteins, cJun exhibits the highest transcriptional activity and the cJun-cFos dimer forms the most stable and active AP-1 complex [68, 69]. There are considerable variations in the activity as well as the expression of AP-1 components, exhibiting cell type or tissue specific activity [75]. Some protein combinations of the AP-1 complexes are inhibitory e.g. JunB and JunD suppress the transformation ability of the cells by forming homodimers or heterodimers with cFos [76–79] and JunB acts as an inhibitor of c-Jun [80].

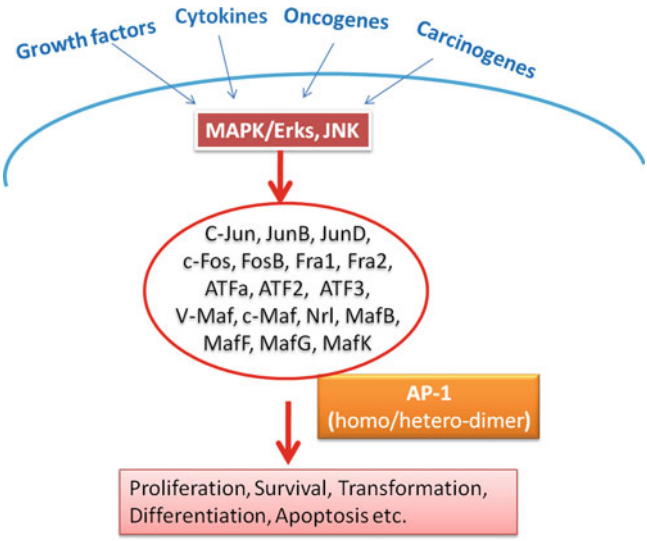

Fig. 6.5 AP-1 signaling: Depending on the stimulus, either homo- or hetero-dimers of different protein components are formed and affect the cellular consequences

6.4.2 AP-1 Activation

AP-1 acts as a mediator to convert vast number of extracellular stimulus by cytokines, growth factors, carcinogens etc. signaling through conserved pathways like transforming growth factor beta, mitogen-activated protein kinases and PI3K into the expression of specific target genes regulating cell proliferation, survival, transformation, invasion, apoptosis etc. (Fig. 6.5). The signaling pathways that predominantly mediate AP-1 activation are the MAPK (mitogen-activated protein kinase) cascade [81, 82] through multiple mechanisms including ERK (extracellularsignal-regulated kinase), JNK (c-Jun N-terminal kinase) and p38 MAPK signal transduction pathways [83]. The precise regulation of AP-1 is critical for maintaining normal functioning of the cell as AP-1 controls both basal and induced expression of genes. AP-1 is stimulated in response to TPA (12-0-tetradecanoylphorbol 13-acetate) and EGF (Epidermal growth factor) and is thus required for TPA or EGF induced transformation [84]. Jun is known to be positively autoregulated by AP-1 [85]. AP-1 dependent transcription is reported to be suppressed by sequestration of c-Fos at the nuclear envelope through interaction with A-type lamins [86]. The phosphatase PTEN is also known to down-regulate AP-1 activity in human glioma cells [23].

Upon activation, the AP-1 protein components dimerize through lucine zipper domains producing a contiguous DNA contact interface that, depending on the dimer (homo/hetero) complexes formed, binds to either TRE (TPA response element) or cAMP response element (CRE) within the promoter region of target genes [87, 88].

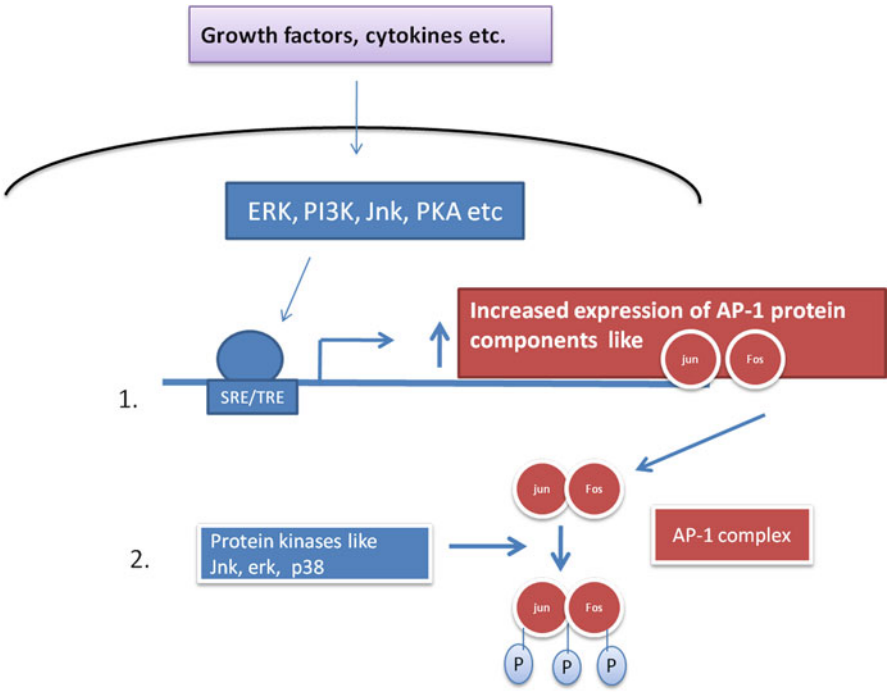

Fig. 6.6 Regulation of AP-1 activation by transcriptional and post-translational modifications

The different dimer combinations of AP-1 regulate the expression of target genes positively or negatively by recognizing different sequence elements in the promoters and enhancers of the target genes and differentially affect the cellular consequences [80, 89].

AP-1 activity may be induced by several mechanisms which may either increase the abundance of AP-1 components or stimulate their activity. AP1 also acts through interactions with protein kinases which causes phosphorylation of the protein complexes and activate them and also interact with a variety of transcriptional coactivators [72]. MAPKs are known to be the major regulators of AP-1 activity which transduce the extracellular signals to activate AP-1 mediated transcription [90].

The ability of AP-1 to transduce diverse signals relies on two features: (1) the overall protein composition either forming homodimer or heterodimer among different proteins of the AP-1 complex [91–94] and (2) the post-translational modification of the protein complex by various kinases (Fig. 6.6) [90, 95, 96]. Phosphorylation at specific sites enhances the transactivating potential of several AP-1 proteins, including c-Jun and c-Fos, independent of DNA binding activities [97].

Tight regulation of the function of c-Jun/c-Fos and c-Jun/ATF2 is a must for preventing the overexpression of the target genes which might lead to pathological changes such as sustained inflammation as well as cell proliferation [72]. The genes encoding AP-1 components mostly behave as “immediate-early” genes, i.e. genes

whose transcription is rapidly induced, independently of *de novo* protein synthesis, following cell stimulation [90].

Target genes known to be regulated by AP-1 are those involved in regulating cell motility and invasion e.g. proteases like MMPs, SPARC (secreted protein acidic and rich in cysteine)), autotoxin, STAT6, IGSF4, ARP 2/3 p41B, CapG etc. [95, 98–103], cell transformation [95, 104]. AP1 also upregulates the expression of various interleukins (IL-1, IL-2, IL-4, IL-5, IL-8), TNFa, GM-CSF, VEGF, COX-2 etc. [5, 105] involved in various inflammatory signaling cascades.

6.4.3 AP-1: Role in Cancer

AP-1 transcription factor is known to play significant roles in tumorigenesis, inflammation, apoptosis, differentiation, and developmental processes [33]. The AP-1 complex is involved in multiple processes linked to tumorigenesis such as proliferation, migration, invasion and metastasis [95, 106]. Increased AP-1 activity has been associated with more aggressive clinical outcome in several cancers including breast cancer [92, 107–109] , prostate cancer [110, 111], keratinocytes [112, 113] and colon cancer [114] etc.

The role for AP-1 in controlling cell proliferation has been proposed based on observations that AP-1 activity is induced upon mitogenic stimulation [104, 115] as well as the reversal of *ras* induced transformed phenotype of keratinocytes by dominant negative constructs of c-*fos* and c-*jun* [82, 113]. The transformation and growth regulatory activity of AP-1 was evident from the experiments studying the effects of overexpression of the components of AP-1 complex in immortalized rat fibroblast [116], Chick embryo fibroblast [117, 118], as well as by blocking the expression of the AP-1 components by antisense/si-RNA or by antibody microinjection of AP-1. AP-1 proteins of c-Fos-JunB complex have also been shown to have tumor-suppressor activity [76, 78, 79].

6.4.4 Role of AP-1 in Linking Cancer and Inflammation

Basal levels of AP-1 activity are important for normal cellular processes like cell proliferation and survival, but over activation of the AP-1 transcriptional activity by extracellular stimulus may increase the expression of AP-1 dependent genes leading to inflammation, angiogenesis, cell proliferation, invasion etc. that drive normal cell into tumorigenesis. AP-1 is one of the major transcription factors involved in mediating inflammatory response and plays an important role in cancer mediated inflammation [82].

The evidence that AP-1 proteins are involved in the inflammatory response of cancer cells is demonstrated in epidermal cells where loss of AP-1 is shown to control cytokine expression through transcriptional and post-transcriptional pathways

[20]. The Jun proteins are also known to control the expression of cytokines and chemokines such as granulocyte colony-stimulating factor, IL-6 and tumour necrosis factor alpha that are involved in inflammatory skin diseases such as psoriasis [75]. Recently the JNK/p38 pathway has been shown to be activated by pro-inflammatory cytokines like TNF-α and IL-1 and may also have a role in inflammatory responses [51]. The promoters of many inflammatory response genes, especially those encoding cytokines and chemokines, have AP-1 binding sites suggesting a possible role of AP-1/JNK pathway in their regulation. JNK is also thought to be involved in the induction of cyclo-oxygenase 2/prostaglandin synthase 2 which plays an important part in the inflammatory response by catalysing the production of prostaglandins [119].

Targeted inhibition of AP-1 activity suggests a pivotal role for AP-1 in oncogenic transformation and progression [106]. Inflammatory stimuli are known to cause activation of AP-1, which in turn induces cytokine expression and cellular immune responses [120, 121]. Hasselblatt et al. [122] had proposed AP-1 as an essential mediator of oncogenic β-catenin signaling in the intestine. Inflammation induced activation of AP-1 pathway has also been implicated in ovarian carcinogenesis [123]. The production of tumor-promoting cytokines by inflammatory cells are known to activate transcription factors such as AP-1 in premalignant cells to induce genes that stimulate cell proliferation and survival [5, 29].

AP-1 also interacts with NF-κB, and a dominant-negative Jun has been reported to inhibit both AP-1 and NF-κB activity in HPV-immortalized human keratinocytes [124]. AP-1 and NF-κB transcription factors are known to be constitutively active in head and neck squamous cell carcinoma cell lines that express pro-inflammatory and pro-angiogenic cytokines [125]. Moreover, the dominant negative constructs of c-*fos* and c-*jun* have been shown to reverse the transformed phenotype induced by activated Ras and also inhibit the invasiveness and tumorigenesis of keratinocytes [113]. The interleukins have been linked to inflammation and subsequent cancer development. Various ILs like IL-1α, IL-1β, IL-6, IL-8 and IL-17 are known to activate transcription factors like AP-1 and NF-κB. IL -1alpha is known to promote NF-κB and AP-1-induced IL-8 expression, cell survival, and proliferation in head and neck squamous cell carcinomas [126]. IL17A is known to induce AP-1 activity and causing neoplastic transformation of JB6 Cl41 cells. Knockdown of AP-1 inhibited the tumorigenesis of MCF7 cell line, pointing to the critical role of inflammatory microenvironment in carcinogenesis [123].

Adding to the complexity of AP1 regulation, a study showed that the knockdown of a trans-membrane protein, FAT1, leads to inhibition of AP-1 mediated transcription and hence pro-inflammatory modulators like COX-2, IL6 and IL1β in glial tumour cell lines [105]. This is apparently mediated via up-regulation of tumour suppressor PDCD4 (Programmed Cell Death4). PDCD4 is a known tumor-suppressor whose expression is decreased under inflammatory tumor promoting conditions. PDCD4 expression is known to be inversely correlated with AP-1 mediated transcription [37, 127–129]. There are several factors responsible for regulating PDCD4 expression including COX-2 inhibitors, miR-21, pAkt etc. PDCD4 levels are known to be induced by cytokines such as IL-12 (interleukin 12), but

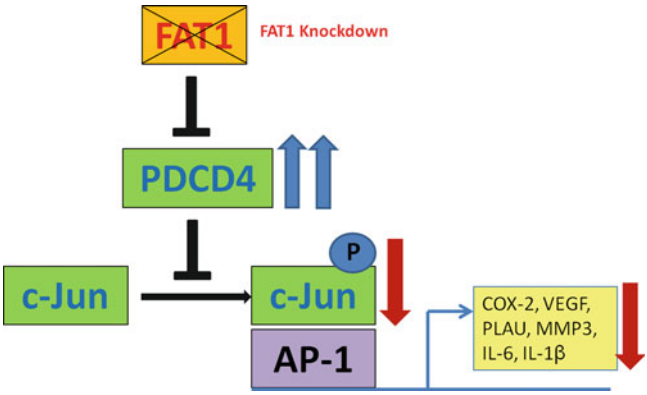

Fig. 6.7 Knockdown of FAT1 led to increased PDCD4 expression, decreased c-Jun expression as well as c-Jun phosphorylation and decreased expression of AP-1 regulated transcripts. From Dikshit et al. [105] reprinted with permission from Kunzang Chosdol and Nature Publishing Group

down-regulated by IL-2 and IL-15 [130]. FAT1, a trans-membrane protein is identified as a novel regulator of PDCD4 expression and consequently AP-1 mediated transcription (Fig. 6.7).

A study by Zhang et al. [131] had identified FAT1 as a differentially expressed gene in human colon carcinoma cells treated with a selective COX-2 inhibitor. Also, AP-1 is known to regulate COX-2 expression via binding to cAMP response element (CRE) [132, 133]. Cyclooxygenase-2 (COX-2) and the prostaglandins resulting from its enzymatic activity have been shown to play a major role in modulating cell growth and development of human neoplasia [134]. COX-2 contributes to tumorigenesis and the malignant phenotype of tumor cells by different mechanisms, including: (1) inhibition of apoptosis; (2) increased angiogenesis; (3) increased invasiveness; (4) modulation of inflammation/immuno-suppression; and (5) conversion of procarcinogens to carcinogens [135].

Thus, AP-1 family of proteins acts as a master regulator of gene expression in response to inflammatory stimuli and oncogenic signal transduction cascades in a wide variety of tumor cell and animal models. It may be considered as an important target for novel anti-cancer therapies. However the ubiquitous nature of its effects may affect the specificity of the response.

6.4.5 Other Members of Activated Proteins (APs)

In addition to AP-1, the other members of activated Proteins (APs) family include AP-2, AP-3, AP-4 and AP-5. The DNA binding motif of all the APs is conserved and binds to 5′-TGANTCA-3′ consensus sequence of the target DNA. Among all activated proteins, AP-1 is the most extensively studied one. AP-2 transcription

factors represent a family of five closely related proteins, AP-2alpha, -beta, –gamma, -delta and -epsilon [136] and are involved in physiological processes, such as morphogenesis and in pathological processes such as tumorigenesis and genetic disease [137]. Recent evidence suggests that the oncogenic [138, 139] as well as tumor suppressive role [140–144] of AP-2 in different cancers, indicating the context dependent effect of AP-2 depending on the ratio and dimerization of AP-2 isoforms present as well as AP-2 modulating factors.

6.5 STAT3

6.5.1 Introduction

STATs (signal transducer and activator of transcription) are members of seven related proteins (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6) containing SH2 domain which helps in homo- or hetero-dimerization during activation [145, 146]. STAT3 (92-kDa protein) is 770 amino acids long with sequential N terminal coiled-coil domain, DNA-binding domain, a linker, SH2 domain, and C-terminal transactivation domain [147]. STAT3 is predominantly present in the cytoplasm and on activation by growth factors and cytokines [148, 149] gets phosphorylated followed by dimerization and nuclear localization where it acts as transcription factor mediating expression of target genes [150–153]. STAT3 is a point of convergence for numerous oncogenic signaling pathways [23] known to upregulate genes involved in cell proliferation, invasion, angiogenesis and inflammation [23, 153, 154]). STAT3 has a role in inducing procarcinogenic inflammatory microenvironment leading to malignant transformation and cancer progression [5].

6.5.2 Mode of Action

STAT3 transcription is triggered by many cytokines and growth factors, including epidermal growth factor [34], platelet-derived growth factor [155], and IL-6 [146] as well as by oncogenic proteins, such as Src5 [115] and Ras6 [156] as well as by carcinogens like cigarette smoke [157] and by tumor promoters [158, 159].

Activation of STAT3 is regulated by various kinases that phosphorylate at tyrosine 705 residue [151, 160–163] and serine 727 residue [164–170] of STAT3 within the C-terminal region. Along with the phosphorylation, acetylation at lysine 685 residue [171] is also critical for stabilization of the STAT3 dimer state and enhancing its transcriptional activity. On phosphorylation and dimerization, STAT3 gets translocated to nucleus, binds to DNA and upregulates the transcription of various genes known to promote cell proliferation, angiogenesis, survival, migration and inflammation (Fig. 6.8) [145, 169, 170].

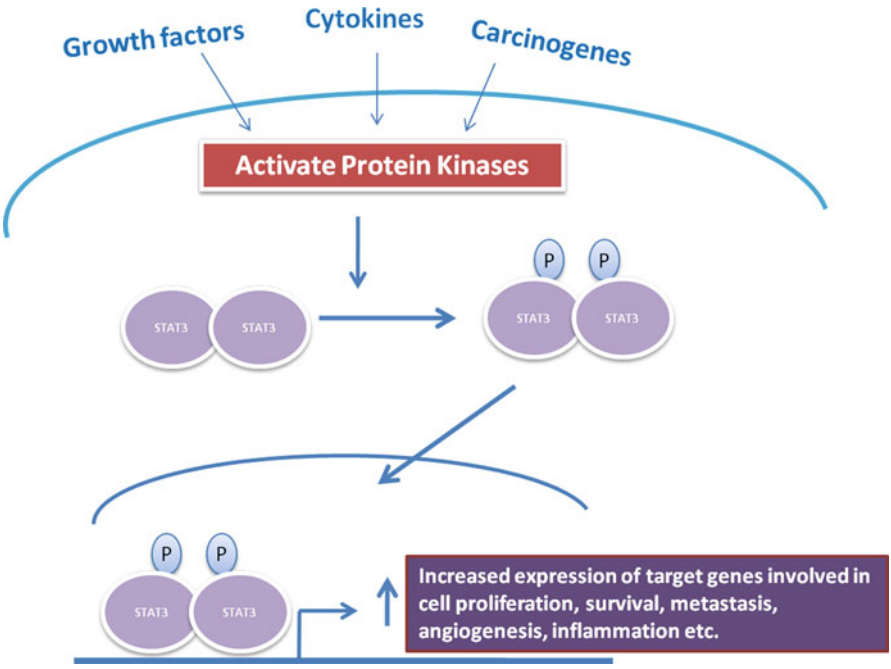

Fig. 6.8 STAT (Signal transducer and activator of transcription)-3 activation and the functional effect in cancer

6.5.3 STAT3 in Linking Inflammation and Cancer

STAT-3 was initially discovered as an acute-phase response protein and a known mediator of inflammation [172]. Most proinflammatory molecules including IL-6, IL-10, IL-17, IL-23 etc., were found to mediate their effects through the activation of the STAT-3 pathway [149]. In some cell types, IL-6-induced STAT-3 activation has been shown to be dependent on cyclooxygenase 2 (COX-2) [173]. Oncogenic transformation in inflammation-associated gastric epithelial cell mediated by cytokine IL-11 and its glycoprotein 130 (gp130) receptor was found to be dependent on increased activation of STAT-3 [174]. Similarly, tumor promoters, lipopolysaccharides, and cigarette smoke also activate STAT-3 pathway [157, 175].

Aside from its role in inflammation, STAT-3 activation by various stimuli are known for transformation of cells [176]. The activation of STAT-3 by src protein kinase [115, 154, 177], by v-Fps; by polyoma virus middle T antigen and v-Sis [161], by human T-cell lymphotropic virus I [162] and by Hepatitis C virus core protein [118] were all found to transform cells. STAT-3 signaling is also required for hepatocyte growth factor/scatter factor-Met-mediated tumorigenesis [178]. STAT-3 is considered an oncogene [154] due to its capability to transform cells and its over activity detected in different tumors.

Constitutive activation of STAT3 promotes tumor cells proliferation and survival [179] and are found to be activated in wide variety of cancers including gliomas [5, 23, 170, 180, 181]. STAT3 mediate expression of various cell survival gene products like bcl-xl [182, 183], bcl-2 [184], survivin [185], Mcl-1 [186] and cIAP2 [187], cyclin D1 [188], VEGF, c-Myc [189]) and pim-1 [190]. Additionally, activated STAT3 is known to suppress apoptosis by suppressing expression of FAS protein [191, 192]). So, Stat3 in many human cancers functions as a critical mediator of oncogenic signaling through transcriptional activation of genes encoding apoptosis inhibitors (e.g. Bcl-x(L), Mcl-1 and survivin), cell-cycle regulators (e.g. cyclin D1 and c-Myc) and inducers of angiogenesis (e.g. vascular endothelial growth factor).

STAT-3 activation plays a major role in tumor cell invasion by regulating the expression of matrix metalloproteinase (MMP)-2 and MMP-1 [119, 193] as well as the expression of the *MUC1* gene [194]. Overexpression of phosphorylated STAT-3 in cutaneous squamous cell carcinoma showed increased invasion and metastasis [182].

STAT3 is found to upregulate the transcription of genes promoting angiogenesis in chick chorioallantoic membrane [137]. Constitutive STAT-3 activity upregulates the expression of VEGF [119, 195, 196] and TWIST [197], promoting tumor angiogenesis and metastasis. STAT3 is also known to regulate EMT through activation of cytokines, including IL-1, IL-6, TNFα, TGFβ [114, 198] and by regulating EMT modulators such as Snail, Twist and ZEB [114, 199].

Overall findings from various studies show that STAT-3 has a central role in regulating cancer-associated inflammation by controlling the expression of various inflammatory modulators and oncogenes, making tumors more aggressive with increased metastatic potential. Role of STAT3 in linking inflammation with cancer is well established as evident from various studies over the past decade in gastric carcinoma, hepatocellular carcinoma, lung cancer, neuroblastoma, multiple myloma etc. [42, 123, 174, 200–206]. So targeting STAT3 in cancers with activated STAT3 form may attenuate tumor progression and invasion as well as it may regulate inflammatory microenvironment of tumors thus making tumors more sensitive to conventional therapy.

6.6 Conclusions

Even with the enormous knowledge and advancement in the field of cancer related inflammation, many questions remain unanswered. Activation of signaling pathways involved in regulating the pro-inflammatory microenvironment in cancers, by the extracellular triggers (either extrinsic or intrinsic) converges onto the common intracellular signaling molecules which eventually stimulate well known transcription factors upregulating the expression of pro-inflammatory cytokines and modulators. As discussed above, the transcription factors NF-κB, HIF1α, AP-1 and STAT3 play enormous role in regulating the expression of genes which are involved in linking cancer and inflammation. In addition to their canonical effect, these transcription factors also cross talk with each other in further maintaining and aggravating the pro-inflammatory microenvironment in cancers (Fig. 6.9) [47, 178, 207].

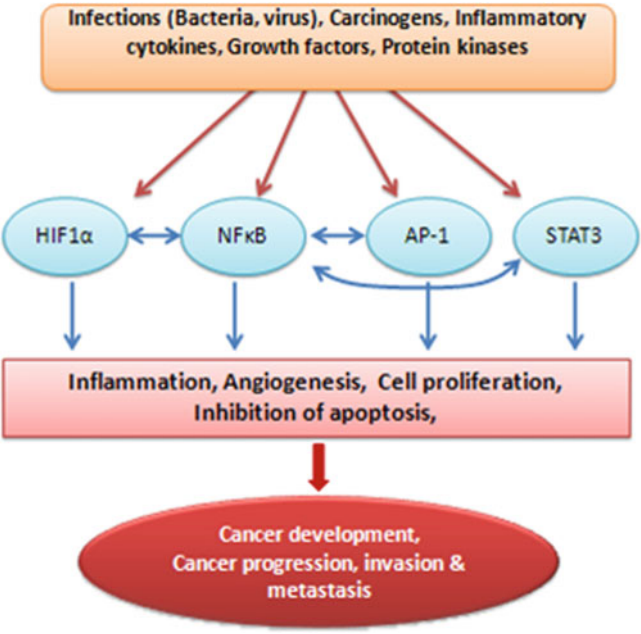

Fig. 6.9 Stimulation of transcription factors having pro-inflammatory and pro-cancerous activities by various stimuli in cancer. Blue arrows depict direct effect of the individual transcription factors, as well as crosstalk among different transcription factors

These transcription factors are the major contributors in regulating the expression of cytokines and inflammatory modulators, and targeting them for molecular therapeutics are considered to be promising with many such drugs undergoing trials. With the identification of these transcription factors in tumor progression and invasion, it has become a major goal for a number of laboratories and companies to develop novel therapeutic molecules so as to target transcription factors for cancer therapy.

Moreover, the mechanisms of action of several old drugs have been re-evaluated and some of them e.g. NSAID were discovered to act, at least, partially through some of these transcription factors. The antitumor activity by NSAID was due to the inhibition of cyclooxygenase (COX) enzyme activity as well as by inhibiting the LPS-induced NFkB-dependent transcription by preventing IkBa degradation [9, 208]. Similarly sulfasalazine was shown to inhibit NF-kB activation [209, 210]. The cardiac glycosides drugs like digitoxin and oleandrin also found to have potential implications in inflammation and tumorigenesis by inhibiting TNF-α-induced activation of NF-kB, JNK and AP-1 [34, 211–214].

Curcumin is another promising anticancer drug, with wide range of effect on cancer and inflammation. Curcumin is known to affect many signaling pathways and found to interfere with the activity of NFkB, STAT3 as well as JNK [196, 215–217]. Recently, in in-vitro and preclinical studies, it has been shown to suppress STAT3

phosphorylation in lung cancer with concomitant reduction in cell proliferation and suggested to be a promising chemopreventive agent in high-risk populations such as smokers [218]. Curcumin as well as other natural chemopreventive agents like retinoid, resveratrol phenethyl isothiocyanate and sulforaphane were found to inhibit AP1 activity [219].

Many novel agents have been identified since then against these transcription factors and are under study at different levels of clinical trials. One such molecule is the bortezomib (proteosome inhibitor) was found to induce apoptosis in a number of otherwise resistant tumor cells, and sensitize such cancer cells to conventional chemotherapies and radiation therapy [158, 220–224]. Other well-known drugs used in cancer inflammation are avastin against VEGF, Rituxan to represses B cells in rheumatoid arthritis & B cell lymphoma and antibodies targeting COX-2 and cytokines like IL-6, IL-1β, IL-8, CCL2 etc.

Along with these, there are drugs targeted exclusively against specific transcription factors which may be used as adjuvant therapeutic molecules in cancer and are under study. These are IKK inhibitor PS1145 and BMS-345541 [225–227], HIF1α inhibitors e.g. echinomycin [3, 55, 228], AP1 inhibitors [94] and STAT3 inhibitors BP-1–102 and WP1066 [229, 230].

The expression of target genes by different protein-complexes of a transcription factor may vary and may not always be same, and cross talk among different transcription factors may also stimulate different subset of target genes. With the result, each tumor type could demonstrate distinct clinical presentations and activities depending on the genes being expressed by these transcription factors. Therefore, for each tumor type, the therapeutic molecules would ideally have to be carefully chosen on the basis of the genetic/epigenetic profile of a patient and the combination of transcription factors being activated. Hopefully, with the explosion in our knowledge and understanding of the biology of neoplasia, personalized therapy is soon likely to be a reality for cancer patients.

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Chapter 7
Regulation of the Jak/STATs Pathways by Histone Deacetylases

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Abstract The regulation of the Jak/STAT pathways involves multiple mechanisms, some of them exclusive to individual cascades, and other shared amongst them. Additionally, there is increasing evidence to support the interconnection of these cascades with other intracellular signaling pathways, which are regulated by independent factors. Thus, the understanding of the Jak/STAT pathway regulation is still growing and recently being revisited to account for the participation of non-conventional regulators. Acetylation of proteins is an emerging regulatory mechanism with demonstrated involvement in the regulation of several intracellular pathways. Specifically, the participation of histone deacetylases (HDACs) is garnering attention due to the feasibility of inhibition of their activity and the subsequent control over the cellular processes being modulated by them. This chapter

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will explore and present the current knowledge about the role of HDACs and their inhibitors in the control of members of the Jak/STAT pathways and direct activators/modulators of their signaling.

Keywords HDACs • STATs • Cytokine regulation • HDAC inhibitors

7.1 Introduction

Cellular responses to the environment are mediated by numerous signaling pathways. The proper reception and translation of these signals allows integration of single cells into a multicellular system where every member has its own function. The Janus Kinase/signal transducers and activators of the transcription (JAK/STAT) signaling pathway is one of the most well studied yet enigmatic integrated signaling systems. This family of pathways' major responsibility is allowing for cellular adaptation to an ever-changing microenvironment. Despite the name, the JAK/STAT pathway contains multiple signaling cascades with a plethora of proteins in addition to the Jaks and STATs.

The initial step in decoding the Jak/STAT pathway was the early finding of inducible genes by IFN treatment achieved almost simultaneously by the Darnell and Stark groups [1, 2]. In the following years these two groups consolidated their findings, identifying and characterizing several components of the Jak/STAT pathway, including several STATs and Jaks [3–5]. By 1994, these reports as well as findings from different groups, lead to the description of the Jak/STATs pathways. Further details about this fascinating race can be found in the article "The JAK-STAT Pathway at Twenty" [6].

The evolutionary conserved Jak/STAT pathway comprises several combinatorial subsets of functional units activated differentially by dozens of cytokines and growth factors. The final cellular responses triggered by these pathways depend on the signal, tissue, and cellular context, and include proliferation, differentiation, migration, apoptosis, and cell survival. Moreover, the JAK/STAT signaling is essential for numerous developmental and homeostatic processes, including hematopoiesis, immune cell development, stem cell maintenance, organism growth, and mammary gland development [7–9].

Some of the regulatory mechanisms affecting the Jak/STAT pathway functionality are those involving post-translational modifications, such as phosphorylation, acetylation, methylation and sumoylation [6]. In particular, this chapter will focus on the current knowledge of protein acetylation as a major modulator of the Jak/STAT pathway. A special emphasis will be assigned to the role of histone deacetylases (HDACs) and their inhibitors in this emerging and expanding field. The protagonists of these pathways will be introduced, and then the participation of HDACs at different levels during the Jak/STAT pathway activation, including their direct participation in formal Jak/STAT pathway members. In addition, their indirect role as regulators of activators of these pathways will be discussed.

7.2 Jak and STATs at a Glance

In a general description, activation of the Jak/STAT pathway begins with the binding of cytokines and growth factors to their corresponding receptors. This, in turn activates JAK proteins, which then introduce specific phosphorylation to either, receptors or STATs proteins on specific residues. The next step involves the homo- or heterodimerization of STATs accordingly with the respective signal sensed by the membrane receptors. This dimerization allows the translocation of STATs to the nucleus, binding to the consensus DNA sequence of 5'-TT(N_{4-6})AA-3' and initiates the transcription of specific target genes [10].

The mammalian JAK family of kinases is composed by four members: JAK1, JAK2, JAK3 and tyrosine kinase 2 (TYK2) [11]. Each of these proteins contains a conserved kinase domain (JH1) and a pseudo-kinase domain at the carboxyl terminus catalytically inactivated (JH2), which has been proposed as a regulator of the former kinase domain [12]. JAKs also have a Src homology 2 (SH2) domain and an N-terminal domain (FERM), which allows its association with cytokine receptors. Also, all members of this family distribute five blocks of sequence similarity throughout the amino-terminal region [13]. JAK1, JAK2, and TYK2 appear to be ubiquitously expressed, while JAK3 expression is normally limited to lymphoid cells.

There are seven mammalian STATs: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6. These proteins are highly homologous in several regions, including a N-terminal domain (NTD), a coiled-coil domain (CC), a beta-barrel DNA binding domain (DBD), a linker domain (LD), a SRC homology 2 (SH2) domain, and a C-terminal transactivation domain (TAD), which is located at the carboxyl terminus (Fig. 7.1) [12, 14]. However, the amino acid sequence diversity and their tissue-specific distributions account for the diverse roles of STATs in response to extracellular cytokines.

Abnormal activation of JAK-STAT pathways has been described in various cancers and immune disorders [8, 15]. These oncogenic processes have been largely described for either Jaks or STATs, and most of them involve constitutive activation. For example, the identification of an activating point mutation in the JAK2 kinase, JAK2V617F, in a vast majority of patients with BCR-ABL myeloproliferative neoplasms (MPNs), provided a significant advance in the knowledge of the molecular pathogenesis of these disorders [16]. This JAK2 mutant activates downstream signaling through the STATs, RAS/mitogen-activated protein kinase (MAPK), and phosphatidyl-inositol 3 (PI3). Noteworthy, treatment with the pan-HDACi LBH589, also known as panobinostat, seems to counteract the constitutive activation of JAK2V617F by inhibition of its autophosphorylation and promotion of its proteasomal degradation. Additionally, panobinostat has shown inhibition in other tumor cell lines such as the human erythroleukemia HEL92.1.7 and Ba/F3-JAK2V617F cells [17].

In most malignancies, it has been found that oncogenic mutations in STATs 1, 3, and 5 lead to persistent tyrosine phosphorylation [18]. Furthermore, abnormal activation of STAT3 has been detected in many cancers, including glioma, melanoma, non-small cell lung carcinoma, head and neck, breast, cervical, ovarian and prostate cancers [19]. The oncogenic property of STAT3 is mainly due to its function as a

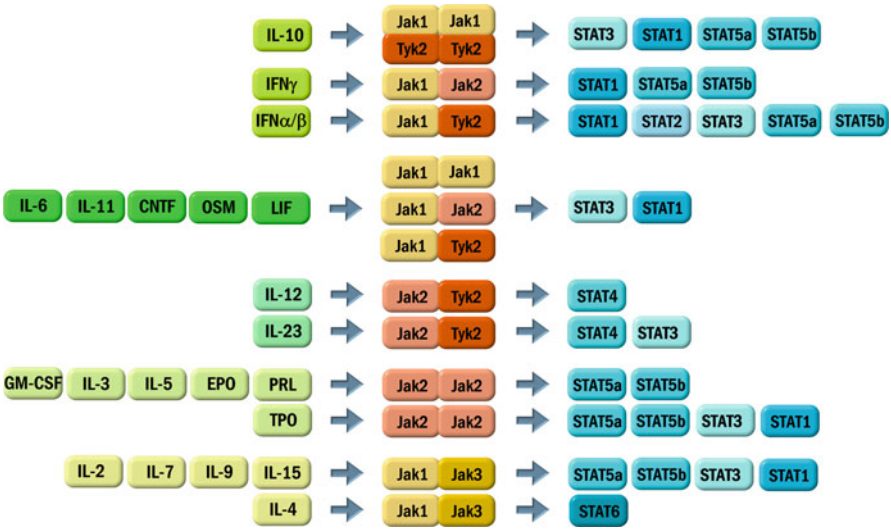

Fig. 7.1 Activation of Jak/STAT pathways by cytokines

modulator of cellular processes such as cell cycle, cell proliferation, and apoptosis by regulation of cyclin D1, Bcl2 and Bcl-xl, genes that ultimately control tumor growth and metastasis [20]. Interestingly, STAT3 can also be abnormally induced by uncontrolled activation of its pathway. For example, paracrine sources of IL-6 can induce autocrine production of IL-6 and pStat3 expression in tumor cells, leading to heterogeneous levels of pStat3 [18].

7.3 Histone Deacetylases and Their Inhibitors

One of the most studied posttranslational protein modifications is the acetylation of lysine amino acids. Initially, these modifications were found at the N-terminal end of histones as a transcriptional regulatory mechanism. Briefly, in a non-modified steady-state, the highly positive N-terminal ends of histones wrap around histones, generating an obstacle for the binding of transcription factors and the recruitment of other proteins that need to read the "writing pattern" on nucleosomes to exert their transcriptional functions. In this context, acetylation of histones neutralizes these positive charges promoting a relaxed nucleosome conformation, and allowing the binding of transcription factors and "writer" proteins. Acetyl modifications are introduced by a heterogeneous group of proteins named histone acetyltransferases (HATs), most of them forming multi-protein complexes that can be selectively recruited to DNA sequences upon exogenous or endogenous cellular stimuli [21]. In opposition, these acetyl modifications can be removed by another group of proteins, HDACs. The 18 HDACs identified in humans are subdivided in two families; the classical HDAC family of zinc dependent metalloproteins, composed by Classes I, II and IV, and the Class III

NAD+-dependent proteins belonging to the sirtuin family of HDACs. The Class I HDACs (HDAC1, 2, 3 and 8) are most closely related to the yeast deacetylase RPD3, and the Class II HDACs are subdivided into Class IIa (HDAC4, 5, 7 and 9) and Class IIb (HDAC6 and 10), both subclasses sharing homology with the yeast deacetylase HDA1 [22]. Finally, the newest HDAC discovered, HDAC11, comprises its own Class IV, and does not share homology with either RPD3 or HDA1 yeast deacetylases. HDAC knock-out mice have severe malfunctions at multiple cellular processes and are in some cases embryonically lethal (HDAC1, 3 and 7) or lethal at the perinatal stage (HDAC2, 4 and 8) [23].

HDACs, originally described as histone modifiers have more recently been demonstrated to modify a variety of other proteins involved in diverse cellular processes non-related to the chromatin environment. This includes deacetylation of multiple non-histone targets, including several proteins involved in cell cycle/apoptosis and immune regulation [23, 24]. The expanded role opens the possibility that the effects of HDACs and HDACi may affect non-epigenetic regulatory pathways, including the Jak/STAT pathway. Indeed, as will be shown throughout this chapter, multiple studies show that this is the case.

A logical approach to elucidating the precise mechanisms of action and functions of HDACs is the use of specific inhibitors that block HDAC enzymatic activity. Some of the compounds that inhibit HDAC activity include butyrates, TSA, trapoxin, SAHA, apicidin, depsipeptide, depudecin, CBHA, MS-275, CI-994, oxamflatin, pyroxamide, scriptaid, CHAP, and valproic acid [25, 26]. Early works indicated that extremely low concentrations of an HDAC inhibitor, TSA, effectively induced cell differentiation and G1/G2 cell cycle arrest. Many studies followed to show that cells cultured with an HDAC inhibitor result in a change in gene expression patterns. A large number of studies have shown that HDAC inhibitors can effectively arrest and counteract transformation of some cells and inhibit the growth of cancers in tumor-bearing animals. In fact, two HDAC inhibitors, Vorinostat and Romidepsin, are FDA approved for treatment of cutaneous T cell lymphoma, and many others HDACi are being pursued in clinical trials at different stages.

7.4 Signal Transducer and Activator of Transcription

7.4.1 STAT1

STAT1 is the principal transcriptional mediator of interferon (IFN) signaling and plays a central role in the regulation of innate and adaptive immune responses. In addition to IFNs, many other stimuli are able to activate STAT1, including cytokines belonging to the gp130 family such as IL-6, LIF, and OSM and growth factors such as EGF and PDGF (Fig. 7.2) [27]. All of these stimuli can also lead to STAT1 phosphorylation in association with other STATs (STAT3 and STAT5) accordingly, with the availability of other STATs and the combination of stimuli received from the environment. Probably the most well documented immunological function of

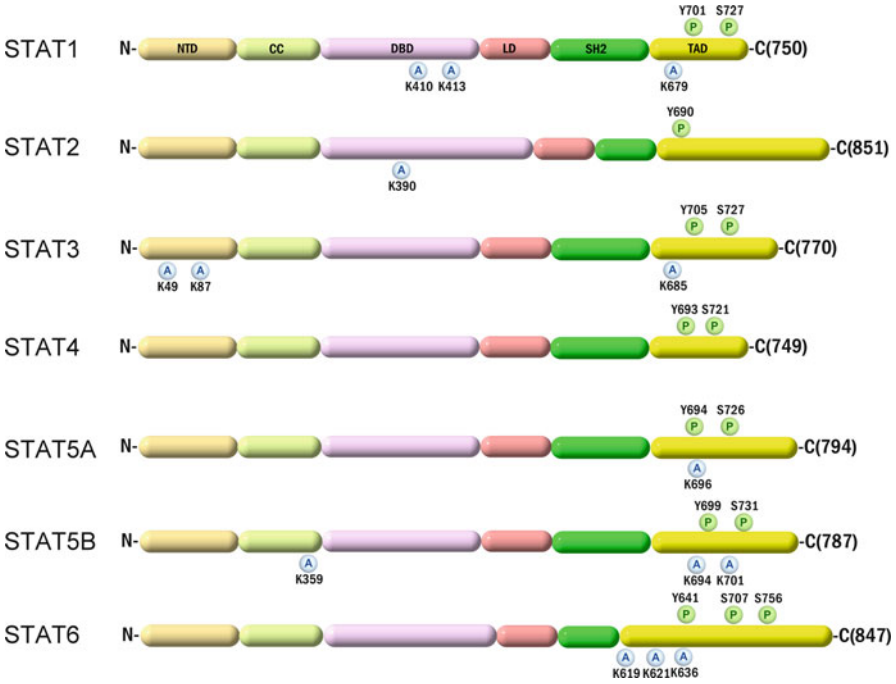

Fig. 7.2 Conserved domains of STATs: Distribution of phosphorylated and acetylated residues

STAT1 is its participation in T cell responses. It is important to mention its positive role in the induction of Th1 differentiation of T-cells, while repressing regulatory T-cells [28]. Additionally, STAT1 is considered as an anti-oncogenic protein. The mechanism by which STAT1 modulates cell death appears to involve both transcription-dependent and independent processes. At the transcriptional level, STAT1 has been described as master regulator of genes involved in the cell cycle and apoptosis such as Bcl-xL [29], p21 [30], iNOS [31], caspases [32], and death receptors [32]. Among its indirect non-transcriptional functions, its association with several pro-apoptotic mediators such as TRADD [33] and p53 [34] are well characterized. An example of the participation of STAT1 in cell death occurs in breast carcinomas, where the p21 activation by STAT1 also involves BRCA1, the most important susceptibility gene known in hereditary breast carcinoma. In fact, BRCA1 and STAT1 synergize to activate p21/waf1 transcription by means of an interaction between the BRCA1 and STAT1 TAD domain. This domain contains serine 727, whose phosphorylation is crucial for the transcriptional activity of STAT1 and which is directly implicated in the recruitment of STAT1 transcriptional coactivators; its mutation causes defective STAT1-BRCA1 binding [35].

The binding of IFN-γ allows the dimerization of its receptors and the reciprocal phosphorylation of Jaks. Once phosphorylated, Jaks will in turn phosphorylate the IFN receptors and provide a suitable STAT1 docking site. While associated with the receptors, STAT1 proteins are phosphorylated on conserved tyrosine 701 by JAKs, allowing the final dimerization of STAT1. There are two well described

phosphorylation sites in STAT1; tyrosine 701 (Y701) and serine 727(S727), both occurring at the TAD domain and triggered by IFN stimulation (Fig. 7.1) [32]. The Y701 phosphorylation within the TAD is required for STAT1 dimerization, nuclear translocation, and DNA binding [36]. The second phosphorylation, S727, seems to be crucial for its transactivational activity, since STAT1 protein mutation at serine 727 has its transcriptional activity reduced by 20 % [37] and fails to recruit transcriptional coactivators [38]. The C-terminal domain TAD has been described as essential for its interaction with other regulatory proteins such as BRCA1 [38], CREB-binding protein (CBP)/p300 family of histone acetyl transferases [39], and MCM5 [40]. The same domain is also a target for the acetylation mediated by CBP, which also has been demonstrated as a STAT1 partner [41].

The magnitude and duration of the events downstream of STAT1 activation are influenced by several intracellular mechanisms and posttranscriptional modifications. In addition to the regulation mediated by phosphorylation, STAT1 can be regulated by acetylation. The first evidence of this mechanism came from early studies in melanoma cells, demonstrating that STAT1 was acetylated in the presence of HDACi [42]. This posttranslational modification was demonstrated to have a negative effect over STAT1 phosphorylation and its subsequent transcriptional activity [43]. The same group also demonstrated that cycles of phosphorylation/acetylation occur after IFN, in addition to other stimuli, treatment. A further hypothesis based on these observations proposes a "pulsing" mechanism of control. Essentially, the sequence of acetylation/phosphorylation observed in activated cells generates a brake for the action of STAT1 over its gene targets. Thus, the acetylation of STAT1 allows its interaction with the phosphatase TCP45 and its subsequent dephosphorylation (Fig. 7.3). This regulatory mechanism promotes a discrete action for STAT1, allowing the cell to escape from death [43].

Several acetylated residues have been described for STAT1 (Fig. 7.1) [44]; however, only a few sites appear to have an impact over STAT1 function. The use of STAT1 mutants has helped to identify two specific residues undergoing acetylation, Lys410 and Lys413 [42]. Interestingly, both residues are located at the DNA-binding domain, suggesting a potential influence in the DNA-binding capability of STAT1 mediated by acetylation/deacetylation.

The first attempts to identify the role of acetylation over the functionality of STAT1 included the identification of the acetyltransferase CBP as responsible for this covalent modification. As well, experiments using HDACi and specific RNAi against HDAC1, 2, 3 helped to identify these HDACs as the counteracting enzymes in this process (Fig. 7.3) [41, 45, 46]. Additionally, a recent report indicates that HDAC4 is also involved in the deacetylation of STAT1 in ovarian cancer cells [47]. The well documented differential tissue distribution of HDAC4 [48] is opening a new perspective about the protagonists in the deacetylation/acetylation of STAT1. It is possible that cell/tissues with low levels of a specific HDAC are less susceptible to the effect of HDACi against STAT1 activity, a hypothesis that is amenable to testing in the near future.

Other groups have demonstrated the role of HDACs over the STAT1 function. For example, Klampfer et al. showed that HDACs are required for signaling by STAT1, and that HDAC inhibitors, as well as small interfering RNA specific for

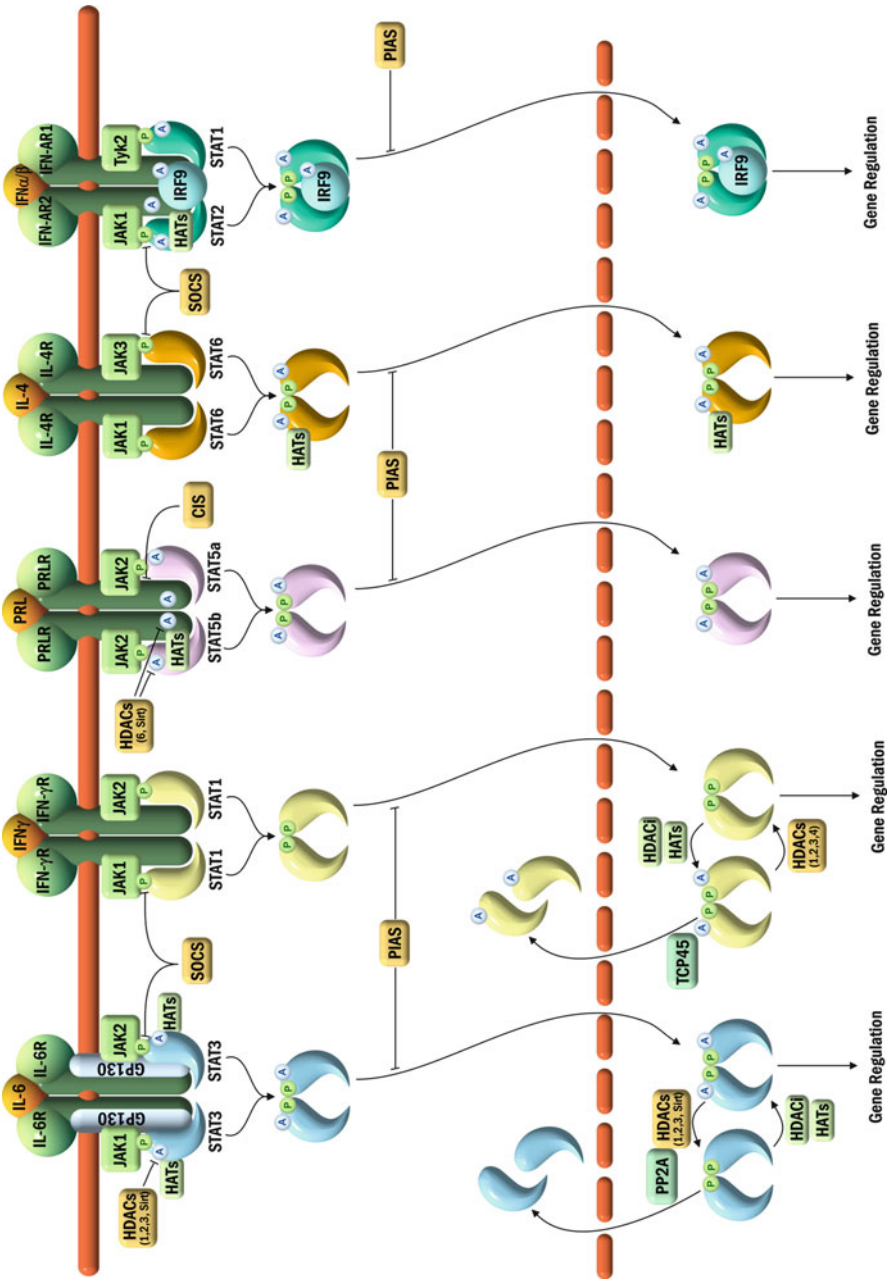

Fig. 7.3 Points of regulation in five classical Jak/STAT pathways

HDAC1, 2 and 3, are able to prevent IFN-γ induced JAK-1 activation, STAT1 phosphorylation and STAT1 dependent gene activation in a colorectal carcinoma cell line [46]. Similar outcomes were observed by TSA treatment in several myeloid cell and tumor cells [49]. Additionally, in colon cancer cells lines treated with the HDACi butyrate, TSA and SAHA, a preferential induction of apoptosis in transformed cells bearing k-RAS mutations was observed [50].

A more detailed functional role of HDACs over the STAT1 functionality and preference for DNA targets has been provided in a recent report [45]. Briefly, the treatment of different cell lines with HDACi counteracts the phosphorylation of STAT1 mediated by IFN. Interestingly, a mutant form of STAT1 replacing both lysines 410 and 413 for glutamine, but not individual mutations, mimicked the effect of a constitutive acetylated STAT1 and the subsequent inactivation of STAT1, suggesting that the number of deacetylated residues controls the functionality of STAT1. The same work also demonstrated that cells treated with IFNα can rescue the negative effect of the double mutant STAT1. A plausible explanation proposed is that IFNα promotes the phosphorylation of other STATs, such as STAT3, that in turn will heterodimerize with STAT1 and allows its recruitment to target DNAs [45].

7.4.2 STAT3

STAT3 modulates the expression of important genes involved in the regulation of a variety of physiological and non-physiological cellular functions, including cell cycle control, cellular differentiation, apoptosis, angiogenesis, metastasis, and innate and acquired immune responses [51, 52]. These genes include IL-17, IL-23, Bcl-xL, Bcl-2, MCL1, CCDN1, VEGF, c-Myc, p53, in addition to others [15, 27]. The Stat3 pathway is activated in response to a wide variety of cytokines such as the IL-6 and IL-10 family of cytokines, GCSF, leptin, IL-21, IL-23, etc. Moreover, STAT3 can be activated by other receptors and signals, including growth factors like PDGFR and EGFR, among others (Fig. 7.2) [15].

In the conventional STAT3 activation pathway (Fig. 7.1), the activation of cell surface receptors by growth factors or cytokines induces the phosphorylation of receptor tyrosine residues allowing the interaction of the STAT3 SH2 domain with the receptor and its subsequent phosphorylation. Phosphorylation of STAT3 can be mediated by the intrinsic tyrosine kinase activity of the activated growth factor receptor or by the Janus kinase (JAK) that associates with activated cytokine receptors, including Gp130. The final activation of STAT3 attained by its phosphorylation at the Tyrosine 705 (Y705) allows its dimerization (hetero- or homo-) and the subsequent translocation to the nucleus to modulate the expression of target genes. Additionally, a second phosphorylation at serine 727 (S727) in the C-terminal transactivation domain allows for the maximal activation of STAT3 target genes [51]. However, some evidence gathered from melanoma models suggests that the phosphorylation of Ser727 is not merely a secondary event and has a role in the survival activity and nuclear translocation of STAT3 in melanocytic cells, and its mechanism of action is independent from the Tyr705 phosphorylation [53].

It is accepted that constitutive activation of STAT3 mediates tumor-promoting inflammation. STAT3 has a dual effect in tumor inflammation and immunity by enhancing pro-oncogenic inflammatory pathways, including nuclear factor-kB (NF-KB) and interleukin-6 (IL-6)-GP130-JAK pathways, and by adding a brake to the STAT1 anti-tumor immune responses, mainly mediated by T cells. Thus, STAT3 has emerged as an attractive target for controlling oncogenic processes due to its critical role in tumor cell survival, proliferation, angiogenesis, metastasis, and immune modulation.

The important role of STAT3 in malignant processes was established after initial studies showed that STAT3 was constitutively activated during v-Src transformation [54], and that its downstream pathway was required for oncogenic transformation by v-Src [55]. Later, other transforming tyrosine kinases, such as v-Eyk, v-Ros, v-Fps, Etk/BMX, and Lck were found to constitutively activate STAT3 in the context of oncogenesis [56]. Moreover, early studies show that the constitutive activation of STAT3 in human breast cancer cells correlates with EGF receptors family kinase signaling and also with aberrant JAK and s-SRC activity [57]. These findings laid the groundwork to understanding the heterogeneous network involved in the activation of STAT3, leading to the discovery of other autocrine and paracrine stimuli influencing the aberrant functionality of STAT3. One of the most well understood signals triggering the oncogenic properties of STAT3 is the cytokine IL-6, which is particularly relevant in multiple myeloma and prostate cancer as IL-6-mediated activation of STAT3 enhances the production of key regulators of cell cycle and apoptosis such as Bcl-2, MCL-1, cyclin D1 and c-myc, which in turn prevents apoptosis and stimulates growth in these tumors [58]. In this context, in a recent report it was demonstrated that STAT3 plays a key role in G1-to S-phase cell-cycle transition through the up-regulation of cyclins D2, D3, A, and cdc25A and the concomitant down regulation of p21 and p27. Thus, constitutive Stat3 activation may lead to a growth advantage of the malignant counterpart [59].

Additionally, STAT3 tumorigenic activation has also been linked to other non-cytokine stimuli, such as EGFR, an event particularly important in lung cancer [60]. Noteworthy, it has been found that STAT3 is a common factor in many oncogenic signaling pathways, and is constitutively activated at a frequency of 50–90 % in diverse human cancers, including multiple-myeloma cells, chronic lymphocytic leukemia, head and neck cancer and many solid tumors such a breast, lung, prostate, ovarian cancers and malignant melanomas [15, 19, 61, 62]. The STAT3 activity is necessary for proliferation and/or survival of many different types of established or primary tumor cells bearing constitutive STAT3 activity, and its inhibition impairs tumor growth in vivo in different types of tumor [56]. Moreover, in murine models it has been reported that STAT3 represses p53 expression by directly binding to its promoter, inhibiting p53 pro-apoptotic activity and contributing to cell survival [63]. In a mouse model of melanoma, it was found that the alternative splice variant STAT3β with dominant negative properties suppressed tumor growth of B16 melanoma cells, supporting the role of STAT3 in these malignancies.

In addition to the well-characterized regulation mediated by phosphorylation, STAT3 can be regulated by acetylation. Interestingly, this mechanism seems to

operate in the opposite direction compared to that observed in STAT1 (Fig. 7.3). Thus, like tyrosine phosphorylation, acetylation is necessary for the STAT3 activation [27]. In this context, persistent acetylation of STAT3 is generally observed in diverse human cancers. For this reason both phosphorylation and acetylation of STAT3 are crucial for STAT3-mediated up-regulation of oncogenic genes [64]. Cytokine-dependent acetylation of STAT3 at the C-terminal lysine-685 (K685) was reported by two independent groups [64, 65]. Both showed that STAT3 undergoes acetylation in various adherent cancer-derived cell lines treated with the related cytokines IL6 or OSM and with the Class I interferon IFNα. In addition to the opposite effect of acetylation observed between STAT1 and STAT3, several other discrepancies have been described. For example, STAT1 has been demonstrated to be acetylated only by CBP [42], and STAT3 can be acetylated by either CBP or p300 [65]. Moreover, the acetylated residues in both STATs are located in different structural domains; STAT1 is mainly acetylated at the residues K410 and K413 located at the DNA binding domain, and STAT3 is acetylated at the K685 located at the transactivation domain. These divergences suggest that the regulatory mechanisms behind these covalent modifications do not affect the same functions.

Early studies showed that HDAC1, HDAC2 and HDAC3 associate with STAT3 and the phosphatase PP2A. Among them; HDAC3 showed the highest deacetylase activity over STAT3. The mechanism proposed points to a facilitation of dephosphorylation of STAT3 mediated by the recruitment of PP2A through the interaction with HDACs [66]. This is further supported by the finding of hyperacetylation of STAT3 in cells treated with the Class I selective HDACi MS-275 [67], and the persistent phosphorylation of STAT3 in cells treated with HDACi [66]. Another body of evidence relating acetylation with the transcriptional regulation mediated by STAT3 involves a potential role in the control of the methylation status of gene promoters. It has been demonstrated that STAT3 interacts with the DNA methyltransferase 1 (DNMT1) [68]. In this context, a recent report showed that the impairment of the K685 acetylation is accompanied by demethylation and reactivation of several tumor-suppressor genes [69]. The same report also identified the potential role of sirtuins in this process through observations that the treatment of cells with the sirtuin activator resveratrol is directly linked to the acetylation status of STAT3 in melanoma cells, and this can reverse aberrant CpG island methylation in melanoma and several other malignancies. The participation of sirtuins in the deacetylation of STAT3 has been also reported by other groups [70, 71], positioning these deacetylases as potential targets in the modulation of the STAT3 activity.

7.4.3 STAT5

Two highly related STAT5 proteins exist: STAT5a and STAT5b. These proteins are encoded by two distinct but chromosomally linked genes with high similarity, although there are functional differences between them, including the DNA binding affinities [72]. STAT5a and STAT5b are activated in response to a variety of

cytokines as well as tyrosine kinase receptors [73]. These include prolactin (PRL), growth hormone (GH), erythropoietin (Epo), trombopoietin (Tpo), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-2, IL-12, IL-3, IL-5, IL-7, IL-9, and IL-15, which are involved in a great many functions regarding cell growth regulation (Fig. 7.2) [74]. The canonical JAK2/STAT5 pathway is one of the most widely studied cellular signaling cascades and is critical for normal hematopoiesis [75]. Moreover, STAT5 has been reported to have oncogenic properties, mainly through its promotion of cell survival and proliferation by tightly controlling the expression of genes involved in cell cycle progression and survival, such as c-myc, bcl-2 and bcl-XL, cyclin D1, thus intervening in the growth control of these cells [76]. Since STAT5 regulates the transcription of cyclin D1/D2 and c-myc in some cell types [77], the constitutive activation of STAT5a/b, probably promotes tumorigenesis by deregulating the cyclin complexes D/CDK4-6, which is responsible for the control progression from the G1 to the S-phase of the cell cycle [56]. Thus, the constitutive activation of STAT5 is a hallmark of hematopoietic malignancies, chronic myelogenous leukemia, erythro-leukemia, acute lymphocytic leukemia, myeloproliferative neoplasms such as polycythemia vera, essential thrombocytopenia [78] and other types of cancer, including breast cancer [79], prostate cancer [80], squamous cell carcinoma of the head and neck, melanoma and hepatocellular carcinoma [81]. Its constitutive activation may also be triggered off by the expression of fusion proteins causing persistent PTK activation, such as JAK2, PDGF-R or ABL [82].

A widely pursued question is whether the STAT5a/b proteins are critical for cellular transformation, as suggested by the presence of activated STATs in a variety of tumor types and the observation that over expression of various forms of dominant-negative STAT5 proteins can partially suppress cell growth. For example, in chronic myelogenous leukemia (CML) the presence of activated STAT5a/b, in the presence of dominant negative can suppress the transformed phenotype [83]. Additionally, considerable attention has been assigned to STAT5a/b as transcriptional regulators of anti-apoptotic genes such as Bcl-X, a characteristic shared with other STATs such as STAT1. Particularly, STAT5a/b dominant negatives have a considerable reduction in Bcl-X levels, which leads to the suppression of cell growth [73].

One of the most studied roles of STAT5 is its participation in the prolactic receptor (PRLR) pathway. The PRLR is an essential type I cytokine receptor involved in mammary gland development during pregnancy and lactation. JAK2 phosphorylates PRLR on multiple tyrosine sites in a cytoplasmatic loop, participating also in the phosphorylation of STAT5a and STAT5b on a conserved tyrosine residue within the C-terminal SH2-dimerization domain. The phosphorylation of STAT5 triggers its dissociation from the receptor and the subsequent dimerization, which activates STAT5 and facilitates its translocation into the nucleus where it regulates gene expression associated with the functions of the ligand prolactin (PRL) [84].

As with other STATs, the activity of STAT5 can be regulated by phosphorylation. Additionally, recent studies in human breast cancer TD47 cells, reported that CBP induces acetylation of STAT5 and, simultaneously, PRLR. The dimerization and subsequent activation of the STAT5 pathway is also enhanced by the pan-HDACi

TSA and the sirtuin inhibitor nicotinamide. These observations were further validated by inhibition of STAT5 after over expression of SIRT2 or HDAC6 [84]. This study also reported the existence of specific sites for acetylation in STAT5b, K359, K694, and K701, as reflected from mass spec analysis and site directed mutagenesis [84]. Interestingly, the residues K694 and K701 are located in close proximity to the phosphorylation site of STAT5, however, their acetylation by CBP was found to occur independently of the phosphorylation on Y699.

In a recent study it was reported that the selective class HDAC6 inhibitors NQN-1 decrease levels of constitutively active STAT5 and attenuated Erk phosphorylation by the acetylation of Hsp90 in human acute myeloid leukemia cell line MV4-11. This demonstrated that inhibition of HDAC6 and the subsequent interference with the Hsp90 chaperone function resulting in the degradation of critical proteins like STAT5, and attenuation of signaling cascades promoting leukemic cell growth [85]. Apparently, STAT5 seems to interact with a variety of HDACs. Thus, another recent report using co-immunoprecipitation and ChIP assays demonstrated that STAT5 is responsible for the recruitment of HDAC1 to the Id-1 gene through direct recruitment to the pro-B-cell enhancer (PBE) regulatory region. This recruitment induces deacetylation of the promoter region of the Id-1 gene as well as deacetylation of the transcription factor C/EBPbeta, whose acetylation diminishes its DNA-binding activity. Therefore, this new function for STAT5 enhances the transcriptional activity of Id-1 [86].

7.4.4 STAT2

STAT2 is mainly phosphorylated and activated by stimuli mediated by IFNα/β, cytokines that activate the ISGF3 complex, which consists of STAT1, STAT2 and IRF9 (Fig. 7.3) [73]. The tyrosine residue Tyr690 of STAT2, required for SH-phosphotyrosine interaction and thus STAT activation, is located near the SH2 domains. Contrarily to other STATs, STAT2 is the only one that does not act as a homodimer, and is the only member of the STAT family that does not bind GAS elements as homodimers [87].

IFNα/β stimulation induces the dimerization of the IFNARs and the subsequent cross-phosphorylation of Tyk2 and of JAK1. Once activated, JAKs phosphorylate the IFNAR2 receptor subunit to generate a docking site for STAT2 and promote its phosphorylation. Nevertheless, IFNAR2 phosphorylation only generates the docking site for STAT2; the subsequent phosphorylation of STAT2 in tyrosine 690, once again mediated by JAKs, will favor binding with STAT1. The binding of STAT1 to IFNAR2 depends on STAT2, but not vice versa [35]. The recruitment of STAT1 to the receptor allows its phosphorylation by JAKs at the tyrosine 701, this permits the release of the heterodimer STAT1/STAT2, which associates with the IRF9 nuclear factor and forms the ISGF3 factor [88], that in turn activates specific target genes within the nucleus recognizing promoter sequences called IFN-stimulated response elements (IRSE) [89, 90]. STAT2 deficient mice are viable and develop normally. However similar to STAT1 deficient mice, STAT2 null mice are susceptible to viral

infections, and cells from these mice are unresponsive to IFNα/β, supporting the essential role that Stat2 plays in the ISGF-3 complex induced by IFNα. In addition, the absence of reduced activation of STAT1 is consistent with the concept that STAT2 facilitates recruitment to, or activation by, the IFNα/β receptor complex [73].

Like many eukaryotic transcription factors, the carboxy-terminal TADs of STAT1, STAT2, STAT3, STAT5 and STAT6 interact with co-activators histone acetyl transferases (HATs) especially p300/CBP. In this context, it has been reported that STAT2 recruits the acetyltransferase protein GCN5 (general control non-repressed), which lead to acetylation of histones in the promoters of IFN-α–regulated genes [13].

All subunits of the ISG3 complex, STAT1, IRF9 and STAT2, are highly acetylated, a process mediated mainly by CBP in IFNα stimulated Hela and 293T cells [87]. The Lys390 within the STAT2 DBD is poorly conserved among other STATs. Although it is unclear whether Lys390 is an evolutionary mistake requiring acetylation for correction or it has a function evolved specially for human cells, STAT2 acetylation on this site may warrant a more flexible interaction between STAT2 and STAT1, allowing STAT1 to interact with IRF9 and DNA within the ISGF3 complex [87]. This potential mechanism has been shown for other transcription factors such as p53, where the acetylation within DBD mediates regulatory interaction with other cofactors without affecting its DNA-binding activity [91]. Whereas acetylation of STAT2 at K390 appears to be required to allow association of STAT2 with IRF9, $STAT2^{K390R}$ constitutively binds STAT1 but fails to recruit IRF9 in IFNα treated cells [44]. The importance of the acetylation of STAT2 was also demonstrated by experiments using the HDACi TSA, which prevent its association with IRF9 and the subsequent recruitment of RNA polymerase II to the ISG54 promoter. Interestingly, ectopic expression of IRF9 reverses the inhibitory actions of TSA, suggesting that IRF9 functions to recruit RNA polymerase II to the promoter of interferon-stimulated genes [92].

7.4.5 STAT6

STAT6 is activated by IL-4 and IL-13 and regulates Th2 differentiation of lymphocytes [73], playing important roles in asthma and other inflammatory lung disease [93]. The absence of STAT6 blocks the differentiation of TH2 cells, and lack of STAT4 impairs IFNγ production by T cells and development of natural killer cells during bacterial and viral infections [13]. STAT6 has been shown to be persistently activated in various hematopoietic malignancies, such as lymphomas and leukemias [94], and upregulates genes important for hematopoietic tumor survival and proliferation when persistently activated in tumor cells [15]. As expected, STAT6 deficient mice lack most of the physiological functions associated with IL-4. In particular, the ability of IL-4 to induce the in vitro differentiation of Th2 cell is lost. This phenotype has provided support for the hypothesis that IL-4, through the activation of STAT6, induces the transcription of the IgE constant region of the heavy chain locus and thereby makes it accessible for the recombinase system [73].

A recent study showed that in several breast cell lines, STAT6 may also be involved in oncogenesis, like STAT1, STAT3 and STAT5. The inhibition of cell growth and induction of apoptosis, reported by other authors in human breast cancer cells after treatment with IL-4 seems to be mediated by STAT6 activation [95]. STAT6 is phosphorylated at low levels on the S707 and 756 serine residues in non-stimulated cells, and IL-4 stimulation triggers higher levels of phosphorylation. These serine phosphorylations occur independently of the Y641 tyrosine phosphorylation in STAT6, which is required for the STAT2 dimerization and subsequent nuclear translocation [35].

In addition to serine phosphorylation, STAT6 can be regulated by acetylation. In fact, STAT6 was the first member of the STAT family to be recognized as being acetylated [96]. However, the real contribution of its acetylation is not completely understood [44].

7.4.6 STAT4

STAT4 is the only STAT that has not been reported to be regulated by acetylation/deacetylation. STAT4 expression is restricted to myeloid cells [97] and is predominantly activated by IL-12, a cytokine produced mainly by APCs that is involved in the differentiation of naive T cells into Th1 cells. In fact, STAT4 and STAT6 are therefore involved in maintaining the equilibrium of the TH1 and TH2 responses, alterations in these proteins cause severe immune disorders, like autoimmune diseases for TH1 and allergic diseases for TH2 [98]. IL-12 is also important in the activation and growth of T cells. It stimulates the production of IFN-γ TNF-α from T and NK cells [99]. The IFNα/β can also activate STAT4 [100] but in a lesser magnitude.

7.4.7 STAT1 and STAT3 Acetylation; Opposing Roles in Survival and Immunogenicity

Although STAT3 and STAT1 control several common targets and share some characteristics, the majority of the cellular events unchained by their activation are opposite; STAT1 inhibits proliferation and enhances both, innate and adaptive immune responses. In the other hand, STAT3 promotes proliferation, survival, and immune tolerance (Fig. 7.4). The activation of these two pathways by cytokine/growth factors is indeed finely tuned by regulatory mechanisms present on each specific cell type, evidencing the intrinsic differences between cells to read and translate signals from the environment. Therefore, the fate of the final cellular outcome will depend on the composition, magnitude and duration during the activation of these two pathways, having a tremendous importance in pathological and pathophysiological conditions such as cancer, autoimmune diseases and immune response against foreign insults.

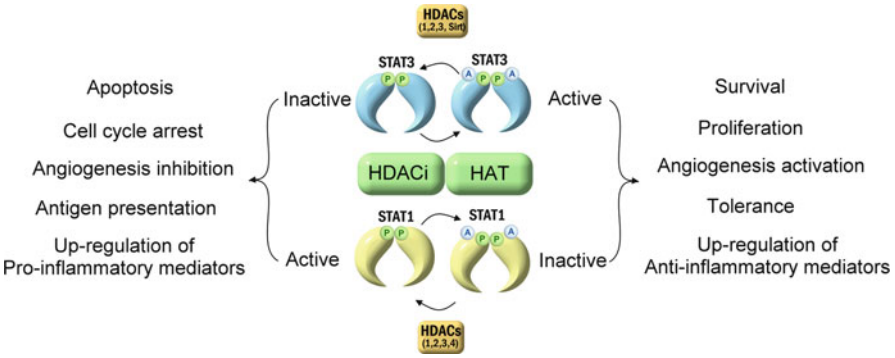

Fig. 7.4 Opposite roles of STAT1 and STAT3 in survival and immunogenicity

As detailed before, the activation of STAT1 is primarily mediated by IFNs. The main function of STAT1 is triggering the expression of pro-apoptotic and anti-proliferative genes [101], and is considered a major barrier in neoplastic proliferation and expansion [27]. In addition to this function, STAT1 is considered a pro-inflammatory mediator due to its capacity to promote the recruitment and activation of immune cells during inflammatory processes. This function is in large part mediated for its capacity to promote antigen presentation by increasing the expression of MHC I and MHC II [27] and its positive regulatory role in the production of chemokines and adhesion molecules such as Mig [102], ICAM-1 [103], CXCL10 [104] and VCAM-1 [102]. A direct proof of the participation of STAT1 in antigenic processes came from early studies using STAT1 -/- and IRF-1 -/- hepatic cells, where the expression of all these mediators was diminished compared with wild type hepatic cells [102]. Additionally, STAT1 modulates the production of COX2 and nitric oxide, important mediators of vasodilatation during inflammatory processes [105].

In the opposite direction, STAT3 is a major regulator of anti-inflammatory events. This role is mainly attributed to its capacity to enhance the production of the anti-inflammatory cytokine IL-10, which in turn will counteract the inflammatory response. Additionally, by recent genome-wide analysis reports, several other target genes potentially responsible for the anti-inflammatory role of STAT3 [106, 107], including Bcl3, Il4ra and Socs3 [9, 10] have been identified. Stat3 can also indirectly ameliorate inflammation by down-regulation of STAT1 target genes, with function principally mediated by IL-10 which interferes with STAT1 phosphorylation [108]. STAT3 also promotes proliferation and inhibits apoptosis by direct regulation of several genes, including the anti-apoptotic factors Bcl-xL [27, 58], Survivin [109], MCL1 [110], and the cell cycle regulators Cyclin D1 [111] and MYC [77]. Additionally, STAT3 facilitates angiogenic events by induction of VEGF [112], HGF [113] and bFGF [114]. Interestingly, STAT1 seems to counteract the effect of STAT3 in angiogenesis by inhibiting the action of VEGF and bFGF [104].

The opposite effects of STAT1 and STAT3 in proliferation and immunogenicity have been explored in different contexts, and outstanding advances have been made

by studies using selective inhibitors and direct genetic abrogation or silencing of their respective expressions [27]. However, new approaches are pointing to dual strategies to control differentially both pathways subsets, for example by manipulation of SOCS regulators [115].

As presented in this review, acetylation/deacetylation is emerging as a regulatory mechanism to control the activation of STATs. Particularly for STAT1 and STAT3 there is evidence indicating that their acetylation status has opposite outcomes in their functions; while STAT3 acetylation is necessary for its activation [64], this covalent modification impairs the transcriptional activation mediated by STAT1 [41]. This duality in the effect obtained by their acetylation status is diagrammed in Fig. 7.4, indicating the opposite outcomes in survival and immunogenicity attained upon acetylation of either, STAT1 and STAT3. This diagram also suggests that finding a common outcome in the function of STAT1 and STAT3 after either acetylation or deacetylation should be expected. Specifically, acetylation of both STATs will tip the balance toward the survival and anti-inflammatory phenotype, and their deacetylation will promote cell cycle arrest/apoptosis and stronger inflammatory responses (Fig. 7.4). This scenario is suggesting that pan-HDACi will favor survival and tolerance; however, in practice, these compounds are promoting exactly the opposite in several malignancies, indicating that the effect of pan-HDACi over other intracellular regulatory processes predominates in the final outcome. Another explanation could be that transformed cells are resistant to this putative regulatory mechanism. This resistance is supported by the fact that several malignancies bear aberrant activation of STATs [8, 15], and perhaps the regulatory mechanisms triggered by acetylation/deacetylation of STATs is bypassed by the hyper-activation of Jak/STATs pathways. Additionally, it is well documented that HDACi are able to decrease proliferation and modify other signaling pathways preferentially in oncogenic cells rather than non-transformed cells [25].

As described before, these two STATs can be acetylated by CBP [44]. However, only STAT3 can be acetylated by p300 [65]. In spite of the well characterized role of HAT complexes in the acetylation of STAT1 and STAT3, only a few HDACs have been tested for the removal of acetyl groups on STAT1 and STAT3. Interestingly, it seems like class I HDACs are able to deacetylate both proteins equally, and only a few reports describe the participation of other HDACs in this process. Noteworthy is the finding that Sirt1 deacetylates STAT3 to inhibit its function [70], and HDAC4 has the same effect over STAT1 [47]. These differences in substrate preferences by HDACs suggests that the differential inhibition of them could be used to differentially inhibit or activate STAT1 and STAT3, a possibility that must be explored in the future.

7.5 Regulators of Cytokine Signal Transduction

As with all biological processes, cytokine signal transduction must be tightly regulated. This regulation in the JAK/STAT signaling pathways is accomplished through multiple families of proteins, which include SH2-containing phosphatases (SHP),

protein inhibitors of activated STATs (PIAS), and suppressors of cytokine signaling (SOCS). An overview of these three families of proteins, their role in regulating the JAK/STAT pathways, and the known interactions of HDACs and their inhibitors with these negative regulatory pathways is given below.

7.5.1 Negative Regulation of JAK/STAT Pathway by SOCs Proteins

The SOCS family of proteins includes SOCS1 through SOCS7 as well as the cytokine-inducible SH2-containing protein (CIS). In addition to the SOCS family proteins listed above, several other protein subgroups that contain the SOCS box, but lack the SH2 domain are sometimes categorizes as SOCS proteins. These include the subgroups: ankryin-repeat-containing proteins, WD-40 repeat containing proteins, SPRY domain-containing proteins, and the RAR-like GTPases. All members of the SOCS family contain a conserved sequence of 40 amino acids referred to as the SOCS box [116]. In unstimulated cells, SOCS proteins are generally expressed at low levels, and become rapidly induced by cytokines, thereby inhibiting JAK-STAT signaling, forming a classic negative-feedback loop [12]. The SOCS proteins are known to achieve signaling suppression through multiple mechanisms. SOC2, SOCS3 and CIS can bind to phosphotyrosine residues on cytokine receptors, thereby competing with STATs for binding sites. SOCS1 binds to phosphotyrosine residues on JAKs, directly inhibiting JAK activity. As well, SOCS can target bound proteins for proteasomal degradation by formation of an E3 ubiquitin ligase complex and subsequent ubiquitination of the bound protein.

SOCS members appear to have overlaps in function, thereby making exact determination of the individual roles difficult. However, knockout studies of SOCS1 mice show increased STAT1 activation and sensitivity to IFN-γ [117]. The SOCS1 promoter is also known to contain an STAT1 binding region; however, it also contains binding sites for STAT3 and STAT6 [116]. In regards to other SOCS: SOCS2 knockout mice have increased signaling by insulin-like growth factor 1, while SOCS3 knockout mice die due to placental insufficiency, a phenotype also seen in STAT3 knockout mice. In macrophages with SOCS3 knockdown, prolonged activation of STAT1 and STAT3 by IL-6 is seen. In addition, an induction of genes associated with IFN-γ signaling is observed [117].

An increase in SOCS expression in cells is dependent on tissue type and the type of cytokine or growth factor stimulation. For example IL-6 stimulation increases mRNA levels of SOCS1, SOCS2, SOCS3, and CIS in liver tissue, while growth hormone stimulation induces expression of only SOCS3 and CIS. In mammary tissue, SOCS2 and CIS are instead induced in response to growth hormone stimulation. As well as tissue and stimulus influencing SOCS expression, expression dynamics are variable. While both SOCS1 and SOCS3 expression liver levels are apparent as little as 20 min after stimulation with IL-6, SOCS1 expression returns to basal levels in 4 h, while SOCS3 take about 8 h. As well, SOCS2 and CIS levels last for 24 h [116].

Currently, little is known about the roles that histone deacetylases play in regulating SHPs, PIASs and SOCs. Indeed, only a few studies to date have been published evaluating the role of HDACi or individual HDACs on these families of negative regulators of cytokine signal transduction. Of these few studies, all of them address SOCS.

The HDACi TSA has been shown to suppress JAK2/STAT3 signaling through its effects on SOCS. In colorectal cancer cells, treatment with TSA led to hyperacetylation of the promoter regions of both SOCS1 and SOCS3, with no effect on the SHP1 promoter. This led to an increase in SOCS1 and SOCS3 expression and a decrease in JAK2/STAT3 signaling. Ultimately, TSA treatment lead to cell cycle arrest and apoptosis in colorectal cancer cells through downstream targets of JAK2/STAT3 signaling such as BCL-2, survivin, and p16 [118]. Treatment with TSA has also been reported to increase SOCS3 expression in N-1 neurons. A similar increase in SOCS3 expression is seen with valproic acid treatment. In contrast, the same study showed that TSA suppressed SOCS3 expression in 3T3-L1 adipocytes [119].

In RAW264.7 macrophages, LPS induces the phosphorylation of the transcription factor ATF-2 as well as the expression of SOCS3. However, when ATF-2 is silenced by siRNA, SOCS3 expression is significantly reduced. Further investigation showed that HDAC1 was found to interact with ATF2 after, but not before, LPS treatment. When treated with TSA prior to LPS stimulation, SOCS3 expression was inhibited. These results indicate that HDAC1 positively regulates AFT2 expression, and thereby positively regulates the expression of SOCS3 [120].

7.5.2 Regulation of STATs by PTPs and PIAS

Several protein tyrosine phosphatases (PTP) have been indicated to regulate JAKs, including SHP1, SHP2, CD45, PTP1B and T-cell PTP (TCPTP). SHP1 and SHP2 are SH2-domain-containing PTPs. Genetic studies indicate that SHP2 is involved in the negative regulation of JAK1 tyrosine phosphorylation after IFN-gamma stimulation is increased [121]. STATs can be negatively regulated by PTPs (such as PTP1B and TCPTP) in the cytoplasm and the nucleus [12], for example the protein tyrosine phosphatase TC45 is responsible for the dephosphorylation of STAT1 in the nucleus [122].

The PIAS family consists of PIAS1, PIAS3, PIASx, and PIASy. As well, there are two known splice variants of PIASx, the alpha and beta forms. PIAS proteins interact with STATs in response to cytokine stimulation, inhibiting the transcriptional activity of STATs. The mechanisms of STAT inhibition vary between the PIAS family members. PIAS1 and PIAS3 are known to directly bind STAT1 and STAT3 respectively, preventing association with DNA. As well, PIAS1, PIAS3, and PIASx can sumoylate STAT1 at Lys-703, close to the site of JAK phosphorylation. However, the impact of sumoylation on STAT1 activity is not understood. Interestingly, PIASx mediated STAT4 repression can be disrupted by HDACi [117]. PIAS proteins have also been implicated in various processes that have no apparent connection to STAT proteins, including induction of apoptosis, modulation of ion channels, interaction with androgen receptors and interaction with RNA Helicase [13].

The SHP family contains two members, SHP-1 and SHP-2. Both of these proteins contain two N-terminal SH2 domains in addition to a C-terminal protein-tyrosine phosphatase domain. These proteins are constitutively expressed and act by dephosphorylating signaling molecules. To achieve this, the SH2 domain of SHPs binds to phosphotyrosine residues on various cytokine receptors or other target molecules. SHP-1 is known to desphosphorylate the IL-4 receptor, c-kit, erythropoietin receptor and JAK2. Conversely, SHP-2 appears to act as a positive regulator of signaling. As well, activation of SHP2 through gp130 requires Jak1. The proliferation depends on STAT3 and SHP2 activation. In addition, SHP2 might be involved in the induction of serine phosphorylation of STAT1 and STAT3 via activation of the MAPK pathway [123]. Interestingly, upwards of 25 % of sporadic juvenile myelomonocytic leukemia patients have mutations in SHP-2 [124].

7.6 Cytokine Regulation by Histone Deacetylases

As cytokine signaling is the initiator of, and cytokine production is often the consequence of the JAK/STAT signaling pathways, discussing the role of HDACs in modulating cytokine expression is essential to understanding their effects on signaling pathways. Histone deacetylases are known to influence expression of a wide variety of cytokines. This knowledge came from several reports utilizing manipulation of specific HDACs and/or pharmacological inhibition of them. A list of the current effects by individual HDACs and HDACi in specific cell types is detailed in Table 7.1. Generally, inhibition of HDACs results in repression of pro-inflammatory cytokines, making histone deacetylase inhibitors (HDACi) attractive therapeutic agents in inflammatory diseases. However, in some cell types and contexts, HDAC inhibition can instead favor inflammatory cytokine production while repressing anti-inflammatory cytokine production [24, 125]. This is the result of the dynamic nature of HDACs with regards to cell type, stages of differentiation and disease context. Most studies to date conducted on histone deacetylase control of cytokine production address the effects of one or more HDACi. The majority of HDACi currently utilized target multiple HDACs, making determination of the role of individual HDACs difficult. However, using recently developed isotype specific HDACi or other experimental approaches to manipulating expression of individual HDACs, a small, but increasing number of studies have identified the role of individual HDACs in the regulation of cytokine production.

7.6.1 Innate Immune Cells

A large number of studies over the past decade have addressed the effect of HDAC inhibitors on leukocyte cytokine production. In a 2002 study, it was demonstrated that administration of the pan-HDACi suberoylanilide hydroamic acid (SAHA), also

Table 7.1 Effect of HDACs and HDACi over the production of relevant cytokines involved in the activation of Jak/STAT pathways

Cytokine	Effect	Condition	Cell type	References
IL-10	↑	HDAC11	Macrophages	[138]
	↑	HDAC1	T cells	[149]
	↑	Valproic Acid	Dendritic cells	[141]
	↑	LBH589	Dendritic cells	[156]
	↑	TSA	Macrophages	[130]
	↑	SAHA	Splenocytes	[157]
	↑	LAQ824	Macrophages	[125]
IL-12	↑	HDAC3	Macrophages	[140]
	↑	HDAC1	Macrophages	[158]
	↑	Valproic acid	Dendritic cells	[141]
	↑	Valproic acid	Macrophages	[132]
	↑	LBH589	Dendritic cells	[156]
	↑	SAHA	Splenocytes	[157]
	↑	TSA	Macrophages	[159]
	↑	Apicidin	Dendritic cells	[133]
	↑	Butyrate	Dendritic cells	[134]
	↑	SAHA	Dendritic cells	[131]
	↑	LAQ824	Macrophages	[125]
IL-6	↑	HDAC3	PBMCs	[160]
	↑	LBH589	Dendritic cells	[156]
	↑	TSA	Macrophages	[130]
	↑	SAHA	Splenocytes	[157]
	↑	Apicidin	Dendritic cells	[133]
	↑	Butyrate	Dendritic cells	[134]
	↑	MS-275	Monocytes	[161]
IFNγ	↑	HDAC1 and HDAC2	T Cells	[162]
	↑	ITF2357	Macrophages	[153]
	↑	TSA	PBMCs	[145]
	↑	TSA	T cells	[146]
	↑	TSA, VPA, NaB	NK cells	[147]
IL-1B	↑	TSA	Macrophages	[130]
	↑	MS-275	Monocytes	[161]
IFNα	↑	TSA	Dendritic cells	[137]
IFNβ	↑	HDAC1 and HDAC8	Various cell lines	[163]
	↑	HDAC6	Various cell lines	[163]
IL-4	↑	TSA	T cells	[164]
	↑	TSA	T cells	[142]
IL-2	↑	TSA	PBMCs	[145]
IL-5	↑	TSA	PBMCs	[145]
IL-23	↑	LBH589	Dendritic cells	[156]
	↑	TSA	Macrophages	[136]

known as vorinostat, to mice results in a reduction of TNF, IL-1β, IL-6 and IFN-γ serum levels after induction by lipopolysaccharide (LPS). Similarly, when human monocytes are stimulated with LPS in the presence of SAHA, there is a pronounced reduction in production of TNF, IL-1β, IL-12 and IFN-γ. As well, IL-18 plus IL-12 stimulation of these cells in the presence of SAHA results in drastic reduction in

IFN-γ production. Additionally, IFN-γ and TNF mRNA levels are reduced by SAHA administration, but IL-8 and IL-1β levels are unaffected. Intriguingly, IFN-γ production by CD3 stimulation is not affected by SAHA treatment [126].

In dendritic cells, pretreatment with SAHA results in a dose dependent reduction in expression of TNF, IL-12 and IL-6 after LPS stimulation [127]. These results are similar with the use of another HDACi, ITF 2,357, also known as givinostat. Furthermore, these reductions are the result of increased STAT3 acetylation [128]. Other studies in dendritic cells show that both SAHA and TSA are also able to modulate the expression of IL-12 and IL-23 [129].

Trichostatin-A (TSA), an HDACi with activity against Class I and II HDACs, has been demonstrated to increase expression of IL-10 in bone marrow derived macrophages. TSA treatment also reduces levels of TNF, IL-6 and IL-1β production in these cells [130]. A separate study demonstrated in both human and murine cells, that treatment of dendritic cells with TSA or SAHA also inhibits production of IL-12p40 post TLR stimulation. Intriguingly, despite decreased expression of protein, HDACi treatment results in increased acetylation of the IL-12p40 locus [131].

In LPS stimulated macrophages, treatment with sodium valproate represses expression of IL-12 and TNF-α while increasing IL-10 expression [132]. As well, in bone marrow derived dendritic cells, apicidin, a fungal metabolite with HDACi properties, inhibits production of IL-12, along with IL-6 and TNF production. Resultantly, treatment of dendritic cells abrogates their ability to produce a Th1 response in T-cells [133]. In agreement, a separate study shows that treatment of LPS stimulated human monocyte derived dendritic cells with butyrate results in a reduction in IL-12p40 as well as IL-6 production. As with apicidin treatment, this impairment results in a deficiency in generating Th1 responses by T-cells [134]. Treatment of LPS stimulated human dendritic cells with the pan-HDACi LBH589 also reduces expression of a variety of cytokines including IL-10, IL-12p70, IL-6, IL-23 and TNF-alpha. Again, the functional consequence of which is impaired activation of T-cells [135].

Interleukin 23 is a heterodimeric cytokine produced by both dendritic cells as well as macrophages. The p40 subunit of IL-23 is shared with IL-12, and like IL-12, the IL-23 receptor signals through the STAT4 pathway. Functionally, IL-23 along with other cytokines is responsible for directing Th17 CD4+ T-cell differentiation. Its role as a pro-inflammatory cytokine and involvement in autoimmunity is becoming increasingly clear. As with various studies in IL-12, IL-23 production is abrogated with HDACi treatment. A study using TSA and SAHA showed human and murine dendritic cells have reduced production of IL-12 and IL-23 when treated with HDACi. Intriguingly, the results are similar when HDACi was given concomitantly with LPS or IFN-γ stimulation as well as administered after stimulation [136].

The Type I interferons are composed of IFN-α and IFN-β. IFN-α is a pleiotropic protein with over twenty known variants. It is mainly produced by innate immune cells in the context of viral infection. There is also evidence of IFN-α having anti-tumor effects. Like IFN-α, IFN-β is generally associated with viral infection and anti-tumor effects. IFN-β upregulates TRAIL expression on CD8+ T-cells. TRAIL upregulation is associated with both anti-viral and anti-tumor responses.

Plasmacytoid dendritic cells are the main producers of type I interferons. In a recent study, a reduction in type I interferons, TRAIL, IL-6 and TNFα was observed after TSA treatment of these cells [137].

While many studies have demonstrated the effects of pan-HDACi or class specific HDACi on cytokine production, more recently the role of individual HDACs in regulating cytokine production has become subject to experimentation. In 2008, HDAC11, the newest discovered HDAC, was discovered to negatively regulate the production of IL-10 in both human and mouse antigen presenting cells. An increase in both IL-10 and IL-12 production by LPS stimulated HDAC11-knockout macrophages is seen, while a decrease in both cytokines is seen in HDAC11-overexpressing macrophages. Interaction of HDAC11 with the proximal promoter region of the IL-10 gene, resulting in deacetylation activity, and thus transcriptional regulation was determined as the mechanism for this regulation [138].

The results of one study show that treatment of mouse macrophages with the pan-HDACi LAQ824 induces chromatin changes at the IL-10 promoter leading to repressed expression of IL-10. Importantly, this study also reveals that LAQ824 treatment results in enhanced recruitment of HDAC11, a negative regulator of IL-10 production, as well as the transcriptional repressor PU.1 to the IL-10 promoter region. Furthermore, an increase in IL-12 production by treated macrophages concomitant to the decrease in IL-10 production is seen. Functionally, in contrast to the described results with other HDACi, this results in increased Th1 activation of T-cells. Additionally, LAQ824 treated macrophages are able to restore function to anergized T-cells [139].

Another study shows that in intestinal macrophages, IL-10 regulates IL12p40 through HDAC3. In these cells, inhibition of HDAC3 by MS275, which target only Class I HDACs, results in increased histone 4 acetylation at the IL-12p40 promoter after IL-10 induction by LPS. As well, shRNA silencing of HDAC3 results in a diminished IL-12p40 repression by LPS induced IL-10 [140]. As in studies described above, another study demonstrated downregulation of IL-12p40 mRNA levels in mouse macrophages after treatment with TSA or SAHA. Additionally, when these cells are treated with the HDACi MS-275, this downregulation is not seen. However, when treated with the HDAC6 specific inhibitor referred to as 17a, a similar reduction in IL-12p40 expression is seen as when treated with TSA [132]. In dendritic cells, it has been recently demonstrated that treatment with valproic acid prior to LPS stimulation decreases the production of IL-10 as well as IL-12p70. These differences are also seen under IFN-γ stimulation. However, the roles of HDAC3 or other specific HDACs were not addressed in this study. Additionally, another study has also demonstrated that HDAC1 mediates repression of IL-12 expression in macrophages [141].

7.6.2 T-Cells

While the above studies have investigated the effects of various HDACi and roles HDACs in leukocytes, several reports have also addressed the direct effects of

HDACi, or the role of specific HDACs, in T-cell cytokine production. In T-cells, as with leukocytes, HDACi treatment often, but not always, decreases expression of pro-inflammatory cytokines.

Results from a study in murine T-cells show that treatment with low dose of TSA and other HDACi results in marked reduction in the production of IL-2. However, sodium butylate treatment does not result in changes in IL-2 production. In addition to its effects on IL-2 production, TSA treatment also results in dramatic decreases in IL-4, IL-13 and IFN-γ production by T-cells. Treatment with both scriptaid and sodium butylate results in changes in only IFN-γ, and to a lesser degree than those seen with TSA. As well, the hyperacetylation associated with HDACi treatment is pronouncedly longer lasting with TSA treatment [142]. Conversely, a study published near the same time reported a TSA induced increase in IL-4 production by T-cells in a murine, collagen induced, rheumatoid arthritis model. This difference may be partially explained by an increase in Th1 cell apoptosis. However, Th2 cells also display an increase in IL-4 gene expression and an associated increase in histone 4 acetylation following TSA treatment [142]. As well, in regulatory T-cells TSA drastically reduces the formation of IL-17 producing cells [143].

Treatment of Jurkat cells with TSA or sodium butyrate elevates production of IL-5, a cytokine responsible for stimulating B-cell growth and antibody secretion as well as the maturation and differentiation of eosinophils. The increase in IL-5 production is associated with increased IL-5 promoter activity and hyperacetylation of both histone 3 and histone 4 at the IL-5 promoter region [144].

A study of whole human peripheral blood mononuclear cells (PBMC) showed ex vivo treatment with TSA results in decreased levels of IFN-γ and IL-2 production concomitant with increases in IL-4 and IL-13 production. When these cells are stimulated with phytohemagglutinin, an activator of T-cells, the cytokine differences resultant from TSA treatment are exacerbated [145]. A separate study also showed that Th1 T-cells when treated with various HDACi including TSA and butyrate have reduced levels of both IL-2 and IFNγ production. Resultantly, treatment with TSA is able to induce tolerance in OT-II ovalbumin-specific naïve CD4+ T-cells [146].

In addition to inhibiting IFNγ production in T-cells and PBMCs, HDACi treatment inhibits production in NK cells. When treated with TSA, valproic acid, or sodium butyrate, NK cells produce lower levels of IFNγ, even upon stimulation with exogenous cytokines such as IL-2 [147].

Class I HDACs are known to complex with Switch independent (SIN3) or Nucleosome-Remodeling protein (NuRD) in T-cells. In proliferating CD4+ T-cells, Sin3-HDAC complexes are recruited to the IFN-γ locus. This complex is displaced by the transcription factor T-bet in Th1 polarization of these cells. Therefore, treatment with HDACi is capable of augmenting IFNγ production by preventing the deacetylation of the IFNγ promoter by the Sin3-HDAC complex. However, as shown above, several HDACi reduce both IFNγ and IL-2 levels. This reduction of IL-2 has been reported to be characteristic of HDACi induced anergy in Th1 T-cells. Furthermore, T-bet has also been reported to indirectly interact with HDAC3 and HDAC5 in respect to GATA-3 binding, a transcription factor driving Th2 polarization [129].

Another study demonstrated that HDAC1 and HDAC2 in complex with Sin3A are displaced from IFN-γ locus in T-cells in Th1 differentiation. When non-stimulated, Th0 CD4+ cells were treated with TSA, there was an acquisition of histone 4 acetylation at the IFN-γ locus not seen in non-treated cells. When these cells were polarized to Th1 cells, no differences in histone 4 acetylation are seen. This increase in histone acetylation was shown to be independent of T-bet and Stat4. Sin3A\HDAC complex is found to associate with several areas of the IFNγ locus, including a known binding site of T-bet, a positive transcriptional regulator of IFNγ, in Th0 cells. When polarized to a Th1 phenotype, association of this complex at the promoter region of IFNγ is lost. However, in both Stat4 and T-bet deficient mice, when T-cells were polarized in Th1 conditions, no loss in Sin3A\HDAC complex at the IFNγ promoter is seen, indicating that dissociation of the complex is dependent on T-bet and STAT4 [148].

A separate group has also reported HDAC1 as a regulator of IL-10 production though E26 transformation-specific 1 (Ets-1). Th1 cells with Ets-1 deficiency had lower levels of HDAC1 recruitment to the IL-10 gene regulatory region and increased IL-10 production. A physical interaction between Ets-1 and HDAC1 was also demonstrated, indicating an Ets-1/HDAC1 synergism in IL-10 transcriptional repression [149].

7.6.3 Disease State Cells

As HDACi are potent modulators of cytokine production, and have a proclivity to down-regulate inflammatory cytokine responses, their use in autoimmune disease contexts is under continued investigation. Highlighting the complex and dynamic roles of HDACs, HDACi are utilized in the seemingly opposite setting as cancer therapeutics, due to their anti-proliferative and sometimes apoptotic effects. Indeed, two HDACi, SAHA and romidepsin, are FDA approved for the treatment of cutaneous T-cell lymphoma. Given this dynamic nature of HDACi, the effects on cytokine production in diseased cells can vary from those seen in healthy cells.

Rheumatoid arthritis is a chronic autoimmune disease with progressive destruction of the joints. Recently, a group has demonstrated that peripheral blood mononuclear cells (PBMCs) from rheumatoid arthritis patients have significantly increased HDAC activity when compared to healthy PBMCs. As well, treatment with TSA reduces levels of TNF and IL-6 production in both healthy and rheumatoid arthritis PBMCs, while treatment with an HDAC3 specific inhibitor, MI192, reduces production of IL-6 in rheumatoid arthritis PBMCs and having no effect on healthy PBMCs [150]. In a separate study, E11 cells, a cell line derived from human rheumatoid arthritis synovial fibroblasts, shows a dose-dependent reduction in IL-6, IL-18 and VEGF production when treated with either MS-275 or SAHA. In addition, treatment with either HDACi results in dose-dependent reduction in IL-1β, IL-6, IL-18 and TNF- α production by an LPS stimulated human monocyte cell line

[151]. A similar study demonstrated that PBMCs or lamina propria cells from Crohn's disease patients produced less TNF mRNA as well as protein when cultured with butyrate and stimulated with LPS. This reduction results from decreased NFkB activation [152].

In a Type 1 diabetes mouse model, the HDACi ITF2357, now known as givinostat, reduces production of IFNγ as well as TNF in peritoneal macrophages and spleenocytes. As well, when insulin-producing cells are challenged with IL-1β plus IFNγ, cells treated with givinostat have a marked reduction in apoptosis [153].

In systemic lupus erythematous, CD4+ T-cells produce high levels of IL-10 and reduced levels of IFNγ. When these cells are treated with the HDACi trichostatin A (TSA), a recovery of cytokine phenotype is seen [154]. In spleenocytes from MRL-*lpr/lpr* mice, a systemic lupus erythematous model, treatment with either TSA or SAHA downregulates expression of IL-10, IL-12, IFNγ, and IL-6. In vivo, TSA treatment of these mice results in a reduction in disease associated symptoms (e.g. proteinuria) [135].

Treatment of the human fibrosarcoma cell line 2fTGH with TSA reduces IFNβ production in response to viral challenge. Using a luciferase report assay and RNA interference, TSA treatment was found to interfere with the transcription factor interferon regulatory factor 3 (IRF3). Utilizing siRNA against individual HDACs, it was found that HDAC1 and HDAC8 both repressed IFNβ expression. However, these results did not explain the reduction in IFNβ production with TSA treatment. Further investigation revealed that HDAC6 enhances IFNβ expression by way of the IRF3 complex [155].

7.7 Conclusions

The Jak/STAT pathways represent one of the most well studied cellular signaling systems. From the initial description of IFN inducible genes, to the describing of the Jak/STAT pathways, the knowledge of these pathways has grown by a staggering amount in the last two decades. Indeed, recently regulation of the Jak/STAT pathway by mechanism beyond phosphorylation, including acetylation, has become evident. At the same time, it is becoming increasingly clear that the Jak/STAT pathways are highly intricate and that much work remains to be done. Additionally, it is also becoming increasingly evident that deregulation of the Jak/STAT pathways plays an important role in disease states, particularly cancer. In the disease context, understanding of not only the Jak/STAT pathways, but also its regulation by cytokines, HDACs, and the influence of HDAC inhibitors are essential to developing more effective and precise therapies. Overall, the Jak/STAT pathways represent a complex family of signaling pathways with equally complex regulation. Future studies will no doubt further unravel these complexities.

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Chapter 8
Receptor Tyrosine Kinases in the Nucleus: Nuclear Functions and Therapeutic Implications in Cancers

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Abstract Receptor tyrosine kinases (RTKs), originally considered as cell surface receptors responsible for transmitting external signals into the interior of cells through diverse signaling cascades, have now been demonstrated to also localize within cells to different compartments such as the nucleus and the mitochondria in addition to their cell surface localization. They also exhibit biological functions when located at the particular cellular compartments. There is increasing evidence indicating that nuclear RTKs, like their cell surface counterparts, are also involved directly in regulating gene expression and DNA repair and replication, leading to cell proliferation, survival, and insensitivity to therapeutic agents. Here, we discuss the advances of our knowledge of nuclear localization of RTKs and their novel functions in the nucleus and describe their implications in cancer therapy.

Keywords EGFR • FGFR • IGF1R • c-Met • VEGFR • Tyrosine kinase inhibitor • Drug- resistance • DNA repair • Gene transcription • rRNA biogenesis

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Abbreviations

ALL	Acute lymphocytic leukemia
BCRP	Breast cancer resistance protein
CBP	CREB binding protein
CCND1	Cyclin D1
ChIP	Chromatin immunoprecipitation
COX2	Cyclooxygenase 2
CTF	C-terminal fragment
DSB	Double-strand breaks
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ERE	Estrogen response element
ERSE	Endoplasmic reticulum stress response element
FGFR	Fibroblast growth factor receptor
ICD	Intracellular domain
IGF1R	Insulin-like growth factor 1 receptor
IR	Insulin receptor
NRG1	Neuregulin 1
PCNA	Proliferating cell nuclear antigen
POMC	Proopiomelanocortin
RSK1	Ribosomal S6 kinase 1
STAT3	Signal transducer and activator of transcription 3
TACE	Tumor necrosis factor-alpha converting enzyme
TKI	Tyrosine kinase inhibitor
TS	Thymidylate synthase
PR	Progesterone receptor
VEGFR	Vascular endothelial growth factor receptor
BAMC	Bovine adrenal medullary chromaffin cells
CML	Chronic myeloid leukemia
HCMEC	Human cardiac microvascular endothelial cells
HDMEC	Human dermal microvascular endothelial cells
HNSCC	Head and neck squamous cell carcinoma
HPEC	Human pulmonary endothelial cells
HUVEC	Human umbilical vein endothelial cells
NSCLC	Non-small cell lung cancer
OSCC	Oral squamous cell carcinoma
SCLC	Small cell lung cancer
SRCC	Sarcomatoid renal cell carcinoma

8.1 Introduction

Receptor tyrosine kinases (RTKs), the high-affinity cell surface receptors for polypeptide hormones, growth factors, and cytokines, are key regulators for important cellular processes, including cell growth, proliferation, differentiation, metabolism, migration, invasion, and survival [1–5]. The activation of RTKs is strictly regulated both temporally and spatially in normal cells, and their deregulation is usually associated with numerous human diseases, including cancer. During the last several decades, 58 RTKs have been identified and classified into 20 different subfamilies. All RTKs share similar molecular structures, including an extracellular region for ligand binding, a single hydrophobic transmembrane helix, and an intracellular region containing a tyrosine kinase domain [1].

RTKs are highly conserved during evolution, and many of them share similar activation mechanisms and common downstream key components. Generally, the binding of ligand to the extracellular domain of a given RTK induces receptor dimerization and activation through trans-autophosphorylation, which recruits cytoplasmic substrates to initiate such downstream signaling cascades as PI3K/Akt, Ras/MEK/ERK, PLCγ/PKC, and JAK/STATs [1, 6, 7]. Following ligand activation, cell membrane-associated RTKs are subjected to rapid endocytosis for degradation in the lysosomes, or recycling back to the cell membrane, or translocation to other organelles such as the nucleus. A number of studies from different laboratories have repeatedly demonstrated nuclear expression of RTKs in cells from either normal tissues or diseased specimens including cancer. In fact, 19 of these 58 RTKs have been detected in the nucleus (Table 8.1). Importantly, the nuclear expression levels of RTKs are associated with cancer progression and correlated with clinical outcome of patients with different cancer types (discussed in further detail below). Consistent with these observations, nuclear RTKs have been shown to play important roles in regulating gene transcription and DNA repair and replication, thereby contributing to tumor cell proliferation, survival, and therapeutic resistance (Fig. 8.1). These novel functions of RTKs in the nucleus are hereinafter referred to as “nuclear functions” to distinguish them from those canonical functions of membrane-associated RTKs. The mechanism of RTK trafficking from the cell surface to the nucleus has been recently reviewed [8–10] and therefore will not be discussed here. In this review, we focus on the nuclear functions of RTKs and their clinical implications.

8.2 Clinicopathological and Prognostic Significance of the Nuclear RTKs

Although the nuclear expression of RTKs in cancer cells has been frequently identified and reported in multiple human tumors (Table 8.1), the clinicopathological import and prognostic significance of nuclear RTKs have not been widely studied. Nevertheless,

Table 8.1 RTKs in the nucleus of various tissues and/or cells

RTK	Tissue/cell types observed with nuclear RTKs	References
EGFR	**Tumor tissues/cells**	[11–18, 41, 42, 45, 46, 49, 50, 55, 57, 60, 61, 63, 64, 123, 126, 165, 181, 184, 194–196, 249]
	Breast cancer, lung cancer, ovarian cancer, esophageal squamous cell carcinoma, prostate cancer, head and neck tumor, gastric cancer, T-cell lymphoblastoma, pancreatic cancer, colorectal carcinoma, bladder carcinoma, pituitary adenoma, nasopharyngeal carcinoma, glioma, choriocarcinoma	
	Normal tissues/cells	[46, 100, 128, 139, 194, 197]
	Keratinocytes, hepatocytes, thyroid tissue, placenta, mouse lung epithelial cells, umbilical endothelial cells	
ErbB2	**Tumor tissues/cells**	[19, 20, 48, 65, 66, 132, 133, 167, 169, 198–201]
	Breast cancer, prolactinoma, colon cancer, ovarian cancer	
	Normal tissues/cells	[100, 139, 202]
	Mouse lung epithelial cells, umbilical endothelial cells, rat Schwann cells	
ErbB3	**Tumor tissues/cells**	[17, 22, 23, 68, 203]
	Prostate cancer, non-small cell lung carcinoma	
	Normal tissues/cells	[67, 100, 139, 144, 202]
	Mouse lung epithelial cells, umbilical endothelial cells, rat Schwann cells, mammary epithelial cells	
ErbB4	**Tumor tissues/cells**	[17, 24, 25, 69, 72–74, 204, 205]
	Prostate cancer, breast cancer	
	Normal tissues/cells	[73, 79–81, 100, 134, 139]
	Mouse lung epithelial cells, umbilical endothelial cells, rat ventricular myocytes, embryonic neural precursor cells, fetal murine type II epithelial cells, hippocampal neurons, breast epithelium	
FGFR1	**Tumor tissues/cells**	[90, 206, 207]
	Medulloblastoma, neuroblastoma, breast cancer	
	Normal tissues/cells	[84–87]
	3T3 cells, BAMCs, neuronal progenitor cells	
FGFR2	**Tumor tissues/cells**	[29, 30, 91, 208–211]
	NSCLC, breast cancer, endometrial carcinoma	
	Normal tissues/cells	[212–215]
	Sertoli cells, immortalized rat osteoblastic Py1a cells, stomach progenitor cells, mammary gland terminal end bud cells	
FGFR3	**Tumor tissues/cells**	[31, 32, 216–218]
	Breast cancer, prostate cancer, bladder cancer	
	Normal tissues/cells	[219]
	Choroid plexus	
IR	**Tumor tissues/cells**	[95, 220, 221]
	Liver cancer, colonic tumors, NSCLC	
	Normal tissues/cells	[94, 99, 137, 145, 222–224]
	Hepatocytes, osteoblast-like cells, corneal epithelial cells, adipocytes	

(continued)

Table 8.1 (continued)

RTK	Tissue/cell types observed with nuclear RTKs	References
IGF1R	**Tumor tissues/cells**	[27, 28, 96, 171]
	Prostate cancer, breast cancer, soft tissue sarcomas, Ewing sarcoma, hepatocellular carcinoma	
	Other diseases	[225]
	Graves' disease	
	Normal tissues/cells	[99, 135]
	Corneal epithelial cells	
VEGFR1/ VEGFR2/ VEGFR3	**Tumor tissues/cells**	[33–35, 226–232]
	AML, ALL, CML, lymphoma, NSCLC, breast cancer, cervical carcinoma, colon cancer, glioma, ovarian granulosa cell tumor, myxoid liposarcoma, HNSCC	
	Normal tissues/cells	[232–238]
	HUVEC, HCMEC, HDMEC, HPEC, cardiac capillary endothelial cells, human thyroid follicular cells, vascular endothelial cells, rodent Brunner's gland epithelial cells	
Met	**Tumor tissues/cells**	[36, 37, 102–104, 239–242]
	Melanoma, uveal melanoma, NSCLC, SCLC, breast cancer, colon cancer, testicular cancer, lymphoma, OSCC	
	Normal tissues/cells	[36, 146, 243]
	Liver cells, melanocytes, colon, skin, testis, lymph node	
TrkA	**Tumor tissues/cells**	[38, 112, 113, 244, 245]
	Pheochromocytoma, glioma, melanoma, colorectal carcinoma	
	Other diseases	[246]
	Parkinson's disease	
	Normal tissues/cells	[114, 247, 248]
	Liver, mouse brain, blastocysts	
Ryk	**Tumor tissues/cells**	
	?	
	Normal tissues/cells	[107, 109]
	Neural progenitor cells	
Ron	**Tumor tissues/cells**	[105]
	Bladder cancer cells	
	Normal tissues/cells	
	?	
Mer	**Tumor tissues/cells**	[136]
	Leukemia	
	Normal tissues/cells	
	?	
Ror1	Using overexpress system to demonstrate Ror1 in the cell nucleus	[110, 111]
c-**Kit**	**Tumor tissues/cells**	[250–252]
	Gastrointestinal stromal tumors, SRCC, extrahepatic bile duct carcinomas	

? unknown

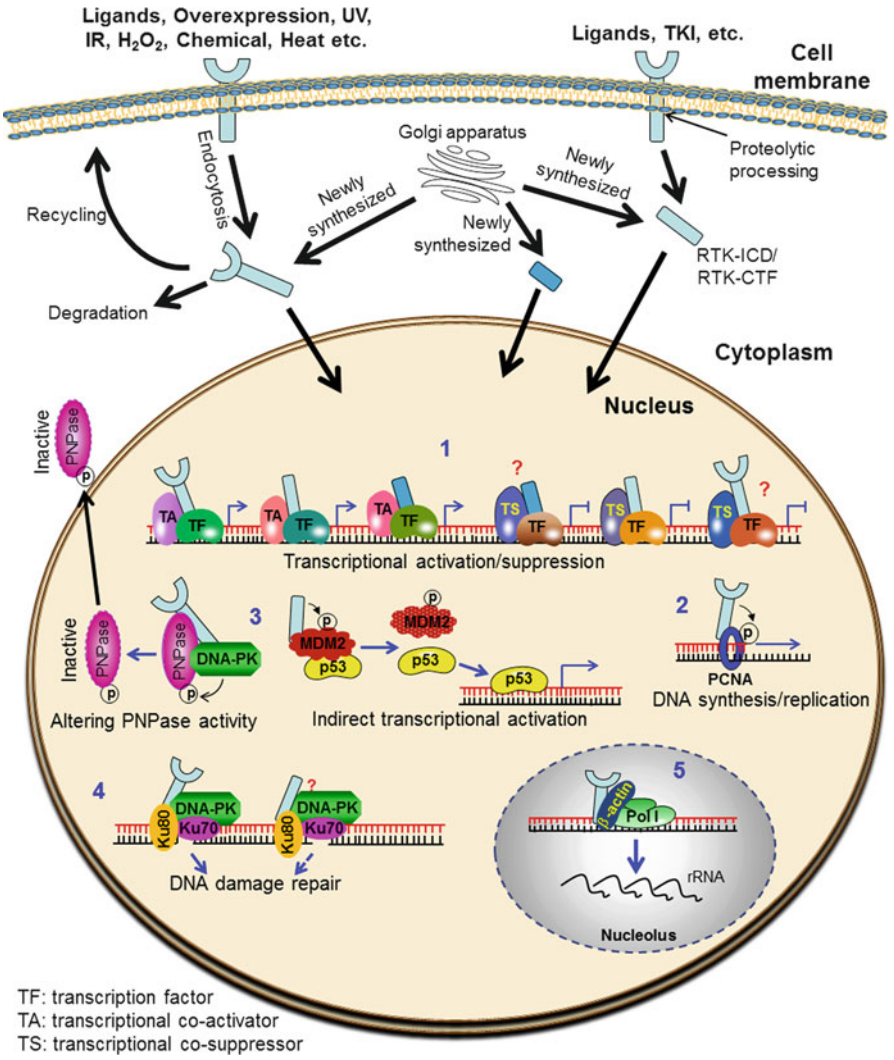

Fig. 8.1 Diverse functions of nuclear RTKs. EGFR and its ErbB subfamily members are the most extensively studied receptors among 58 RTKs and therefore used to depict the functions of nuclear RTKs. Upon ligand stimulation or other stimulation, cell surface RTKs (including in-frame deletion variants such as EGFRvIII) can undergo endocytosis. RTKs, in addition to traditional degradation and recycling, can translocate into the nucleus either directly or after proteolytic cleavage first to release their intracellular domains (ICD) (e.g., ErbB4-ICD). In addition, newly synthesized RTKs (e.g., FGFR1) or RTK variants either from in-frame splicing (e.g., mLEEK, nucErbB3) or from alternative translation initiation (e.g., ErbB2-CTFs) can go to the nucleus directly without any stimulation. Once in the nucleus, nuclear RTKs or RTK variants can (*1*) associate with nuclear transcription-related proteins such as transcription factors and/or transcriptional co-activators or transcriptional co-suppressors to regulate gene transcription; (*2*) interact with and phosphorylate DNA replication-related protein(s) such as PCNA to modulate DNA synthesis; (*3*) interact with nuclear kinase/phosphorylase protein(s) such as MDM2 or PNPase to change the phosphorylation status of nuclear kinase/phosphorylase protein(s), leading to alteration of their stability/activity; (*4*) interact with DNA-PKs and other DNA repair complex proteins to regulate DNA damage repair; (*5*) associate with β-actin and Pol I in the nucleolus to stimulate rRNA biogenesis; The scale of the diagram does not reflect the relative sizes of different molecules or subcellular structures

existing studies have shed light on the importance of nuclear RTK expression as a prognostic indicator for clinical outcome of human cancers. Among these RTKs, EGFR subfamily receptors have been the most extensively studied to date.

The first report from an immunohistochemical analysis of EGFR in 130 breast carcinomas indicated that 37.7 % of tumor samples were positive for nuclear staining of EGFR, among which 6.9 % had high expression levels (by staining). Interestingly, the high expression level of nuclear EGFR rather than cytoplasmic EGFR in cancer cells was found to be significantly correlated with poor overall survival of cancer patients [11]. Consistently, another analysis of 113 breast carcinomas detected nuclear EGFR expression in 40 % of cases, and among these12 % had high nuclear EGFR staining [12]. In addition, nuclear EGFR correlated not only with tumor size, lymph node metastasis, and Nottingham prognostic index but also with shorter overall survival [12]. Similar to the findings in breast cancer, positive nuclear EGFR staining was also detected in 24.3 % of 37 cases of oral squamous cell carcinomas, and those with high nuclear EGFR expression showed a tendency toward poor survival [11]. Psyrri et al. analyzed 95 oropharyngeal cancer cases by quantitative immunohistochemistry and found 33 cases (35 %) with high nuclear EGFR staining and 34 with low nuclear EGFR expression. Patients with high nuclear EGFR expression also had a higher local recurrence rate and inferior 5-year disease-free survival than patients with low nuclear EGFR expression [13]. In patients with esophageal squamous cell carcinoma, nuclear expression of phosphorylated EGFR (i.e., the activated form) was identified in 19 (36.5 %) of 52 cases, which correlated significantly with TNM stage and lymph node metastasis as well as with poor prognosis of cancer patients [14]. The prognostic value of nuclear EGFR has also been corroborated in ovarian cancer. High nuclear expression of EGFR was found in 28.3 % of cases in a cohort of 221 ovarian cancer tissues, and a significant inverse correlation between high level of nuclear EGFR and overall survival was shown [15]. Recently, overexpression of nuclear EGFR was found to be significantly associated with advanced AJCC (American Joint Committee on Cancer) stages and vascular invasion in gallbladder carcinomas [16]. This study also showed that phosphorylated EGFR, which is predominantly overexpressed in those cases marked by both vascular and perineurial invasion, significantly correlated with adverse disease-specific survival [16]. Similar to wild-type EGFR, the study of EGFRvIII, a constitutively active mutant of EGFR, in a cohort of 74 cases from patients with matched hormone-sensitive and hormone-refractory prostate tumors revealed that nuclear EGFRvIII expression in hormone-refractory tumors was associated with quick death from biochemical relapse and therefore correlated with poor overall survival [17]. However, the prognostic value of nuclear EGFR reported in the cancers mentioned above was not found for colorectal cancers, in which nuclear EGFR expression did not associate with any clinicopathological characteristics tested, including tumor grade, tumor size, and tumor stage. However, a high percentage (57 %) of strong nuclear EGFR expression was identified and significantly related with CCND1, an important indicator for cell proliferation [18]. Taken together, these findings indicate that high expression of nuclear EGFR may be a useful clinical prognosticator for human cancers.

The clinical relevance of ErbB2 nuclear expression in human cancer was first noted in studies of breast cancer. Dillon et al. first reported that nuclear ErbB2 expression was associated with tumor size and tumor grade in a cohort study of 560 breast cancer patients. In addition, nuclear ErbB2 expression level was significantly correlated with poor 5-year disease-free survival [19]. This finding was confirmed by a recent study using a tissue microarray containing 273 primary invasive breast carcinomas in which nuclear expression of ErbB2 was detected in 33.6 % of tumor samples and identified not only as a significant independent predictor of worse overall survival in patients with ErbB2 membrane overexpression but also as a biomarker of lower overall survival in patients with tumors that overexpress membrane ErbB2 and lack steroid hormone receptors [20].

Although few reports are currently available on the clinical relevance of nuclear ErbB3 in human cancers, one study indicated that the expression of ErbB3 in prostate cancer cells depends on androgen status and bone microenvironment and is dynamically regulated during prostate cancer metastasis in in vivo xenograft models [21]. Moreover, a predominant nuclear staining of ErbB3 was identified in 50 % of 24 lymph node/bone metastatic specimens expressing ErbB3 in a total 45 human prostate cancers, implying a positive link between nuclear translocation of ErbB3 and prostate cancer metastasis. Another study also showed that nuclear ErbB3 expression is more frequently identified in prostate cancerous tissues than in normal or benign prostatic hyperplasia tissues, and the increased nuclear ErbB3 staining was associated with increasing Gleason grade of prostate cancers. Among prostate cancer tissues, nuclear expression of ErbB3 was more frequent in hormone-refractory (17/17, 100 %) than in hormone-sensitive (37/92, 40.2 %) tissues [22]. Interestingly, a 3-year biochemical-recurrence-free survival probability study from the same research group later showed that a low frequency of nuclear ErbB3 is associated with increasing biochemical recurrence in patients with prostate cancer and positive surgical margins after radical prostatectomy [23]. Therefore, the prognostic value of nuclear ErbB3 in human cancers seems to be much complex.

Similar to ErbB2, the clinicopathological significance of nuclear ErbB4 was investigated primarily in breast cancer. Both the full-length ErbB4 and proteolytically cleaved ErbB4 (ErbB4 intracellular domain; ErbB4-ICD) were found in the nucleus of cancer cells, and their expression in the nucleus was linked to poorer survival outcome [24, 25]. In contrast to nuclear ErbB4, the prognostic value of total ErbB4 in breast cancer is much more controversial [26], highlighting the significance of nuclear ErbB4 expression in clinical prognosis.

The prognostic value of nuclear IGF1R on disease outcome is still inconclusive among studies from different cancers and therefore needs further investigation. Nuclear expression of IGF1R was identified in 48 % (94/195) of clear cell renal cell carcinomas and associated with adverse prognosis [27]. However, a recent study in sarcomas indicated that the high percentage of nuclear IGF1R expression (75 % in 16 cases) was significantly correlated with better progression-free and overall survivals upon IGF1R antibody therapy [28].

In lung squamous cell carcinomas, nuclear expression of FGFR1 has a significant correlation with worse recurrence-free survival while nuclear expression of FGFR2is strongly related with worse recurrence-free and overall survival [29].

Similar to the findings in lung cancer, nuclear FGFR2 expression in breast cancer was also correlated with worse overall and disease-free survival [30]. In contrast to FGFR2, the prognostic value for nuclear FGFR3 expression seems to be controversial. During the development from normal urothelium to bladder cancer, FGFR3 translocates from the cytoplasm to the nucleus [31], suggesting its potential role in bladder carcinogenesis. However, a small cohort study of 55 primary bladder cancers via immunohistochemical staining identified nuclear expression of FGFR3 in 32.7 % of bladder tumors, and patients with tumor cells positive for nuclear FGFR3 expression had less tendency to have recurrence within 2 years after primary bladder cancer treatment [32].

High nuclear expression of phosphorylated VEGFR2 was reported in VEGFR2-positive ovarian granulosa cell tumors, and this high level nuclear expression was significantly correlated with the increased number of blood vessels in tumor tissues and inclined to a positive association with tumor recurrence ($p = 0.006$) [33]. In addition, nuclear expression of phosphorylated VEGFR2 was also detected in 87.4 % of AML cases and 65.8 % of ALL cases and closely correlated with increased microvessel density [34]. However, the prognostic value of nuclear phosphorylated VEGFR2 in acute leukemia remains uninvestigated. In contrast to VEGFR2, the nuclear expression of VEGFR3 seems to be a predictor for better survival in patients with NSCLC [35].

The clinicopathological feature of nuclear c-Met is variable among different cancer types. For example, a high level of nuclear c-Met (a truncated form) expression was significantly correlated with poorer 5-year survival in a cohort study of 640 cases of invasive breast cancer [36]; however, it seemed to have a good prognosis in oral squamous cell carcinomas in a small cohort study of 55 cases [37].

Despite the reported discrepancies in the clinicopathological significance of nuclear RTKs, such as IGF1R, FGFR3, and c-Met, cancers with high nuclear expression of RTKs are inclined to have unfavorable prognosis. Given that multiple forms of RTKs might exist (e.g., full length vs. truncated form and wild type vs. constitutive activated mutant in the case of EGFR), it remains to be seen if they might contribute to these discrepancies. Therefore, to fully understand the clinical importance of nuclear RTKs in human cancers, specific antibody to distinguish different forms of RTKs in the nucleus and more studies with large cohorts of samples will be required. Elucidating the functional roles of RTKs in the nucleus will not only help us understand the clinicopathological significance but also explain the discrepancies in the prognostic value of nuclear RTK expression among different cancer types.

8.3 Functions of RTKs in the Nucleus

In the 1980s, several growth factors and their receptors were detected in the nucleus and found to associate with chromatin [38–43]. For example, nerve growth factor (NGF) receptor was shown to associate with *Eco*RI-digested chromatin [39]. Another study demonstrated that ^{125}I-EGF was taken up by cells into the nucleus to bind to chromatin [43]. Interestingly, the uptake and subsequent binding of ^{125}I-EGF

to chromatin are receptor dependent and correlated with the expression level of EGFR, indicating the possibility of EGFR localization in the nucleus. This idea was supported by a later study that specific mAb targeting EGFR, like EGF ligand, is able to bind a chromatin-associated receptor when taken up by cells and localized in the nucleus [41], suggesting EGFR was associated with chromatin in the nucleus. Soon afterwards, EGFR was confirmed in the nucleus of hepatocytes by both immunoelectron microscopy and nuclear isolation followed by immunoprecipitation [44], and EGF/EGFR complex was detected in the nucleus through ^{125}I-EGF crosslinking to EGFR [45]. Moreover, nuclear EGFR was demonstrated to functionally induce some nuclear proteins' phosphorylation in response to EGF [46]. In 1994, ErbB2, another EGFR family receptor, was unexpectedly found to have transcriptional activity and be able to translocate into the nucleus [47]. Together, these studies provide a rationale for the phenomena of growth factor receptors' association with chromatin in the nucleus and open a new direction for possible roles of RTK in the nucleus. The functions of nuclear RTKs, however, remained largely forgotten until 2001 when the first genomic target of nuclear EGFR was identified and the mechanism of how EGFR transactivates CCND1 gene expression elucidated [45]. Since then, more and more RTKs have been shown to have functions in the nucleus (Table 8.2). Indeed, investigation of the nuclear functions of RTKs has gradually received more attention than ever before and has become an increasingly attractive and important research area in human cancer.

8.3.1 Regulation of Gene Transcription

8.3.1.1 Transcriptional Activation by ErbB Family Receptors

The neu-encoded p185 protein, a rat homologue of human HER2/ErbB2, was the first RTK shown to have nuclear function, namely transcriptional activity, in 1994 [47]. However, the first physiological relevant transcriptional target (COX2) of HER2/ErbB2/neu was not identified until a decade later [48]. EGFR was the first RTK found to have nuclear function in cancer cells [45], and its nuclear function is the most well-characterized among all nuclear RTKs. Similar to the rat neu-encoded p185, the C-terminus of EGFR contains transactivation function, and EGFR binds to an AT-rich consensus sequence (i.e., ATRS) of CCND1 gene promoter to stimulate its transcription [45, 49–51]. CCND1 is a key regulator for cell-cycle progression, and its overexpression is usually related to tumorigenesis. The pathological significance of nuclear EGFR-stimulated CCND1 gene expression was further validated by a positive correlation between nuclear EGFR expression and CCND1 protein level in several cancer types [11, 12, 15, 16, 18]. EGFR has been identified as a DNA-binding protein through an unbiased protein-DNA interactome analysis [52]. However, because EGFR does not contain any known DNA-binding domains, it is plausible that nuclear EGFR binds the specific dsDNA through other nuclear protein(s) that have DNA-binding domains. This

Table 8.2 Diverse functions of RTKs and their associated proteins in the nucleus

RTK	Detected form in the nucleus	Possible nuclear functions	Target genes/proteins	Nuclear-associated proteins	References
EGFR/ErbB1	Intact EGFR	Transcriptional regulation	CCND1↑	RHA	[45, 53]
			iNOS↑, STAT1↑, COX2↑, c-Myc↑	STAT3	[54–57]
			b-Myb↑	E2F1	[58]
			Aurora A↑	STAT5	[59]
			BCRP↑		[62]
			TS↑		[63]
		DNA repair		DNA-PK, rH2AX	[119, 122, 125, 177]
		DNA replication		PCNA	[115]
		Protein activity regulation		DNA-PK, PNPase	[161]
	EGFR VIII	Transcriptional regulation	STAT1↑, COX2↑	STAT3	[55, 56]
		?	?	STAT3	[196]
	mLEEK	Transcriptional regulation	GRP78↑, UPR↑	?	[64]
	EGFR-ICD	?	?	?	[253]
ErbB2/HER2	Intact ErbB2	Transcriptional regulation	COX2 ↑, PRPK↑, FANCC↑,	?	[48]
			CCND1↑	PR, STAT3	[65]
			TS↑		[63]
			rRNA↑	β-actin, RNA Pol I	[140]
			ErbB2/HER2↑	mccroH2A1.2	[66]
		DNA repair		?	[133]
	ErbB2-CTF/P95L	Therapeutic resistance to TKI			[167, 169]
ErbB3/HER3	Intact ErbB3	?	?	?	[21–23, 144, 202]
	nucErbB3	Transcriptional regulation	Ezrin↑, HMGB1↓	?	[67]
	ErbB3-80 kDa	Transcriptional regulation	CCND1↑	?	[68]

(continued)

Table 8.2 (continued)

RTK	Detected form in the nucleus	Possible nuclear functions	Target genes/proteins	Nuclear-associated proteins	References
ErbB4/HER4	ErbB4-ICD	Transcriptional regulation		YAP	[71]
			β-casein↑, Sftpb↑	STAT5A	[78, 79]
			ERE-element gene↑, PgR↑	ER	[24, 73]
			P53 target gene↑	Hdm2	[76]
			Eto 2-mediated gene transcription↓	Eto-2	[75]
			GFAP↓, S100β↓	Tab2 and N-CoR	[81]
			HIF-1α-mediated gene transcription↑	HIF-1α	[74]
			Others		[80]
	Intact ErbB4	DNA damage response		?	[134]
FGFR1	Intact FGFR1	Transcriptional regulation	C-Jun↑, CCND1↑	?	[85]
			FGF2↑	?	[84]
			NF-L↑, NSE↑		[86]
			TH↑	RSK1 and CBP	[87, 206]
			TH↑, FGF2↑, FGFR1↑	Nurr1, Nur77, RXR, RAR	[88, 89]
	Intracellular C-terminal FGFR1	Transcriptional regulation	KRTAP5-6↑, PRSS27↑, SFN↑. EBI3↓, GRINA↓	?	[90]
FGFR2	Intact FGFR2	Transcriptional regulation	Myc↑	PR and STAT5	[91]
FGFR3	Intact FGFR3	?	?	?	[31, 32, 217, 218]
	FGFR3 intracellular domain	?	?	?	[254]
IR	Intact IR	Transcriptional regulation	GCK↑, EGR-1↑	IR signaling complex	[95]
			Malic enzyme↑	30 kDa transcription factor	[94]
			IGF-1R↓	?	[96]

IGF1R	Intact IGF1R	Transcriptional regulation	IGF-1R↑	?	[96]
			CCND1↑, Axin2↑	LEF1	[98]
			Binding to enhancer regions of chromatin to activate transcription	?	[97]
IR/IGF1R hybrid	Intact forms	Transcriptional regulation	Affecting a clustering genes by genome-wide ChIP sequence	?	[99]
Met	Intact Met	?	?	PAX5	[102]
	Met C-terminal fragment (60 kDa)	Transcriptional regulation	Transactivation of Gal4Luc	YAP	[103]
				β-catenin	[104]
Ron	Intact Ron	Transcriptional regulation	c-Jun↑, FLJ46072↑, SSTR1↑	nEGFR	[105]
VEGFR1	Intact VEGFR1	?	?	?	[231, 232, 255]
		ICD acting as transcriptional activator in yeast two-hybrid system	?	?	[256]
VEGFR2	Intact VEGFR2	Transcriptional regulation	VEGFR2↑	SP1	[106]
VEGFR3	?	?	?	?	[35, 228, 236]
Ror1	Intact Ror1	Transcriptional regulation	RDX↑, EZR↑, SOS2↑, CALD1↑	?	[111]
Ryk	Intracellular domain	Transcriptional regulation	Dlx2↑, Olig2↓	?	[109]
Trk A	Intact TrkA	?	?	ERβ	[114]
Mer	Intact Mer	Chromatin bound with unknown function	?	?	[136]

? unknown; *nEGFR* nulcear EGFR; *up arrow* up-regulation; *down arrow* down-regulation

hypothesis is consistent with mounting evidence showing that most RTKs including EGFR mediate gene transcription in the nucleus through their associated nuclear partners such as transcription factors/cofactors (see Table 8.2). For example, transactivation of CCND1 gene expression by nuclear EGFR has been demonstrated through its association with RHA, a transcription cofactor with AT-rich dsDNA binding ability [53]. Moreover, nuclear EGFR also interacts with STAT3, E2F1, and STAT5 to activate the transcription of genes such as iNOS [54], COX2 [55], STAT1 [56], c-Myc [57], b-Myb [58], and Aurora A [59] in different cancer cells. Nuclear EGFR may be also involved in stimulating POMC transcription in pituitary adenomas [60]. Consistent with the roles of these genes in regulating tumor cell growth, survival, and invasiveness, nuclear EGFR is linked to cancer cell proliferation in vitro [49, 50] and tumor progression in vivo [60, 61]. Moreover, nuclear EGFR has been recently demonstrated to contribute to tumor cell drug resistance by activating the expression of genes such as BCRP [62] and TS [63], further substantiating the importance of EGFR nuclear translocation in cancer progression. However, it is not yet clear whether EGFR mediates BCRP and TS gene expression through its interaction with the nuclear factors mentioned above or with other unknown factors. In addition to wild-type EGFR, the constitutively active variant of EGFR, EGFRvIII, can transactivate STAT1 and COX2 genes in cancer cells by interacting with STAT3 in the nucleus [55, 56]. Interestingly, mLEEK, a newly identified in-frame splicing variant of EGFR that lacks extracytoplasmic, transmembrane, and tyrosine kinase domains, has been reported to also translocate into the nucleus and act as a transcriptional activator to bind ERSE DNA motif and regulate the expression of endoplasmic reticulum stress-related genes such as GRP78 in cancer cells [64].

As previously mentioned, COX2 was the first characterized genomic target of nuclear ErbB2. ErbB2 binds to the HER2/ErbB2-associated sequence (HAS) of COX2 gene promoter to activate its transcription [48]. Supporting this mechanism, a strong positive correlation between nuclear expression of ErbB2 and COX2 protein levels was observed in human primary tumors [19, 48]. Both nuclear ErbB2 and COX2 were later shown to be predictors for poor disease-free survival in breast cancer patients on endocrine treatment [19]. Upon progestin stimulation, PR induces nuclear translocation of ErbB2 to promote the assembly of nuclear STAT3/ErbB2/PR transcriptional complex in which ErbB2 acts as a coactivator of STAT3 [65]. This complex then binds to the CCND1 promoter through STAT3 to stimulate CCND1 expression and therefore increases breast cancer cell growth [65]. In addition, ErbB2 increases its own gene transcription in the nucleus by specifically interacting with atypical histone variant mH2A1.2 to enhance the binding of mH2A1.2 to ErbB2 promoter, thereby facilitating ErbB2 mRNA expression [66]. Moreover, this feedback stimulation of ErbB2 transcription by nuclear ErbB2 itself is dependent on its own kinase activity. This finding is clinically supported by a high correlation between the mRNA expression of mH2A1.2 and ErbB2 as observed in both breast cancers and ovarian cancers through human tumor microarray analysis [66]. However, the transcription factor(s) involved in this process remains unknown.

Although the role of full-length ErbB3 in regulating gene transcription in the nucleus has not yet been reported, two ErbB3 variants have recently been discovered to function in gene expression in the nucleus [67, 68]. An alternative transcript of ErbB3, corresponding to the intracellular domain of full-length ErbB3 with a molecular weight of 80 kDa (ErbB3-80 kDa), was identified in cancer cells and found to mainly localize in the nucleus where it binds to CCND1 promoter to activate CCND1 expression in a heregulin-independent manner [68]. Another nuclear ErbB3 variant with a molecular weight of 50 kDa (nucErbB3), which is generated by an alternative transcription initiation containing of exons 23–28 of full-length ErbB3 but with an independent 5'-UTR, was recently identified in rat Schwann cells as a transcriptional cofactor in the nucleus. Through ChIP-chip and microarray assays, nucErbB3 was shown to regulate the expression of 63 genes, including Ezrin and HMGB1 [67]. However, whether these nuclear variants (nucErbB3 or ErbB3-80 kDa) mediate gene transcription directly by binding nuclear ErbB3 to DNA or indirectly by interacting with other nuclear cofactor(s) is still unclear. In addition, the functional role of nucErbB3 in human cancers remains to be elucidated.

It is generally accepted that, after a two-step successive proteolytic processing by TACE and γ-secretase, an 80-kDa intracellular domain of ErbB4 (ErbB4-ICD) translocates into the nucleus and executes its nuclear functions to regulate cell proliferation, differentiation, and apoptosis [26, 69, 70]. The association of ErbB4-ICD with transcriptional coactivator YAP enhances the ability of nuclear ErbB4 in gene transactivation [71]. Upon E2 treatment, ErbB4-ICD increases the expression of genes containing ERE half-site such as PR, SDF-1, and ErbB4 itself through its interaction with ERα to stimulate a growth-promoting signal in breast cancer cells [72]. This finding was supported by the observation of a significant correlation between PR and nuclear ErbB4-ICD in human ER^+ invasive breast ductal carcinomas [73], highlighting the oncogenic effect of nuclear ErbB4-ICD in ER-mediated breast cancer development. Moreover, nuclear ErbB4-ICD might also promote tumor progression through its interaction with and stabilization of HIF1α, therefore leading to increasing HIF1α target gene expression in breast cancer cells [74]. ErbB4-ICD also associates with the transcriptional corepressor ETO2 (a tumor suppressor) in the nucleus and blocks its transcriptional suppression function independently of ErbB4-ICD kinase activity [75]. Therefore, ErbB4-ICD may contribute to tumor progression through its inhibiting tumor suppressor ETO2-mediated gene suppression. In contrast to its function in promoting tumors, nuclear ErbB4 has also been shown to activate the genes involved in cell differentiation and apoptosis, which may lead to tumor growth suppression. For example, heregulin induces the association of ErbB4-ICD with Hdm2 to stimulate Hdm2 tyrosine phoshporylation and ubiquitination and consequently stabilizes p53 and increases expression of its target gene p21 [76]. Nuclear ErbB4-ICD, through its interaction with and activation of STAT5A, was found to increase the expression of β-casein and surfactant protein B (Sftpb) in normal epithelial cells, leading to cell differentiation and maturation [77–79]. In addition, a recent study found that the expression of 47 genes, including PFN1, NEDD4, and DCTN4, in hippocampal neuronal cultures was upregulated by ErbB4-ICD upon NRG1 stimulation [80]. Given that most of these

genes are involved in both neuronal function and schizophrenia, it is reasonable to believe that nuclear ErbB4-ICD may contribute to normal and schizophrenic neuronal function. On the basis of these results, it has become increasingly clear that nuclear ErbB4-ICD-mediated gene expression is complex and may be cell type- and cell context-dependent.

8.3.1.2 Transcriptional Inhibition by ErbB Family Receptors

In addition to their transactivation function as mentioned above, ErbB family receptors also play an important role in suppressing gene expression through association with nuclear co-repressors. nucErbB3 has been shown to regulate the transcription of both Ezrin and HMGB1 genes in rat Schwann cells. Interestingly, nucErbB3 stimulates Ezrin promoter activity while suppressing HMGB1 transcription [67]. Although the mechanisms for the different regulation effects of nucErbB3 remain uninvestigated, it is conceivable that nucErbB3 may interact with different nuclear factors to execute its different functions in gene transcription. ErbB4-ICD, on the other hand, was shown to associate with N-CoR through TAB2 to form ErbB4-ICD/TAB2/N-CoR transcriptional suppressor complex, which moves into the nucleus to repress the expression of genes (e.g., GFAP and S100β) to prevent differentiation of neuronal progenitor cells [81].

8.3.1.3 Transcriptional Functions of Other RTKs

FGFR Subfamily

Like EGFR, full-length FGFR1 can enter into the nucleus and bind to the nuclear matrix [82]. Nuclear FGFR1 also localizes to the nuclear speckles, which are associated with the sites of RNA synthesis and processing. These data indicate that nuclear FGFR1 may act as a direct and global transcriptional regulator [83]. The first target gene of nuclear FGFR1 is FGF2. In bovine adrenal medullary chromaffin cells (BAMCs), angiotensin II (AII), a peptide hormone, stimulates its membrane receptors to induce tyrosine phosphorylation of nuclear FGFR1, which then binds to and transactivates the FGF2 promoter through an AII-response element at the FGF2 promoter [84]. Nuclear FGFR1 has also been shown to increase the transcription of c-Jun and CCND1, suggesting a possible role for FGFR1 in regulating cell proliferation [85]. The expression of neurofilament-L (NF-L) and neuron specific enolase (NSE) genes can also be activated by nuclear FGFR1 during cAMP-induced neuronal differentiation, indicating that nuclear FGFR1 may also have a role in cell differentiation [86]. In 2005, nuclear FGFR1 was shown to upregulate tyrosine hydroxylase (TH) and FGF2 expression through its interaction with CPB and RSK1 to activate CPB by releasing CPB C-terminal domain from RSK1 inhibition. Interestingly, the function of nuclear FGFR1 in activating TH and FGF2 expression that results in cell differentiation is independent of its kinase activity [87]. Recently, nuclear FGFR1 was further

shown to associate with nuclear receptor transcription factors (such as RXR, RAR, Nur77, and Nurr1) to stimulate TH, FGF2, and its own gene expression in embryonic stem cells (ESCs) to induce cell differentiation [88, 89].

A strong link between nuclear FGFR1 and human cancers came from a recent study that showed that granzyme B-cleaved FGFR1 (molecular weight 55 kDa, with tyrosine kinase domain and C-terminus of FGFR1) after activation by its ligand translocates to the nucleus to regulate the expression of several genes responsible for cell migration in breast cancer cells [90]. For example, genes (KRTAP5-6, PRSS27, SFN, EBI3, GRINA) regulated by the 55-kDa nuclear FGFR1 were shown to be involved in cell migration. Knockdown of KRTAP5-6, PRSS27, and SFN, which are upregulated by FGFR1, decreased tumor cell migration whereas knockdown of EBI3 and GRINA, which are downregulated by FGFR1, increased tumor cell migration [90]. Importantly, the presence of nuclear FGFR1 in the invading cells in both human tumor tissues and a three-dimensional culture model of breast cancer [90] highlights the clinical importance of this nuclear FGFR1 in tumor progression.

Similar to FGFR1, FGFR2 also localizes in the nucleus to transactivate gene expression in breast cancer cells [91]. Treatment of cells with medroxyprogesterone acetate (MPA) and FGF2 induces FGFR2 translocation into the nucleus to form a complex with PR and STAT5, and the PR/FGFR2/STAT5 complex then activates the progesterone response element (PRE)- and gamma activated site (GAS)-containing reporter genes. Furthermore, this complex was found to increase the expression of Myc gene through binding to the PRE sequence of the Myc promoter. Forced expression of constitutively active FGFR2 increased tumor cell proliferation in vitro and tumor growth in vivo (mouse model) [91]. Colocalization of FGFR2, PR, and STAT5 was confirmed in human tumor tissues [91]. Taken together, these findings suggest that nuclear translocation of FGFR2 contributes to breast cancer progression.

IR Subfamily

As early as 1995, nuclear IR was proposed to function as a gene expression regulator based on its structure analysis, which showed that the insulin receptor α-subunit contains several zinc finger-like motifs and an RGG box [92]; therefore, a model of an insulin/IR/RB complex to regulate gene expression was hypothesized [93]. This hypothesis has not been yet validated, but a later study showed that nuclear IR induces malic enzyme gene expression through regulating the phosphorylation of insulin response element (IRE) transcription factors in hepatocytes from mice treated with glucose meal [94]. More recently, IR and its associated signaling complex, SHC/Grb2/SOS, were found to bind to the same insulin-inducible gene loci (e.g., EGR-1 gene locus) to increase transcription upon insulin stimulation [95], indicating that the gene regulation mediated by nuclear IR is much more complex than previously expected. In addition, IR was found to bind to the IGF1R promoter to downregulate IGF1R expression in ER-depleted C4.12.5 cells (derived from MCF7) due to a possible competition between IR and limited ER for Sp1 transcription factor [96]. In contrast, IGF1R binds to its own gene promoter to increase its

own gene expression in such ER-depleted C4.12.5 cells, suggesting an autoregulation loop in ER-negative breast cancer cells [96]. Using electrophoretic mobility shift assays (EMSAs) with randomly synthesized dsDNA probes and ChIP-based cloning strategy, Larsson's group found that nuclear IGF1R binds mostly to the enhancer regions of chromatin to regulate gene transcription [97]. Moreover, a recent study demonstrated that nuclear IGF1R associates with β-catenin and transcription factor LEF1 to increase the promoter activity of LEF1 target genes, such as CCND1 and axin2, in several human cancer cells [98], further supporting the important roles of nuclear IGF1R in cancer biology. Interestingly, another study detected a hybrid receptor of IGF1R and IR (IGF1R/IR hybrid-R) in the nucleus of human telomerized corneal epithelial (hTCEpi) cells. Genome-wide ChIP-sequencing assay with antibodies against IGF1R and IR identified 52 target genes for IGF1R and 31 genes for IR; of these 52, 11 genes are involved in cell proliferation/cell cycle control and six are related to cell death/apoptosis [99]. This study suggested that IGF1R or IR translocates into the nucleus as an IGF1R/IR heterotetrameric complex that, once in the nucleus, could co-regulate corneal epithelial homeostatic pathways. One interesting question to be addressed is what the different functions are between their nuclear homodimer receptors and heterotetrameric complex. In addition, it is worth noting that heterodimers among ErbB family receptors are common phenomena and have been detected in the nucleus [100]. It would be of interest to further investigate whether these heterodimer receptors of ErbB family proteins have a similar transactivation function in the nucleus as that of IGF1R/IR.

Met Subfamily

PAX5 is a nuclear transcription factor that is believed to promote cancer cell survival and metastasis and which is highly expressed in SCLC cells but not in NSCLC cells [101]. In addition, PAX5 was found to be the main regulator of c-Met (full-length) expression in SCLC cells [102]. Interestingly, phosphorylated full-length c-Met was shown to interact and colocalize with PAX5 in the nucleus of HGF-treated SCLC cells [102]. However, the functions of phosphorylated full-length c-Met and its association with PAX5 in the nucleus of SCLC cells has yet to be determined. In the highly aggressive MDA-MB-231 breast cancer cell line, a C-terminal fragment of c-Met (Met-CTF) rather than the intact c-Met is the major form of c-Met identified in the nucleus and is constitutively activated independently of HGF. In addition, Met-CTF was shown to have transcriptional activity when the C-terminal Met fragment containing the juxtamenbrane domain was fused with GAL4-DNA binding domain in a Gal4Luc assay, and its gene transactivity was further enhanced by YAP [103]. Furthermore, this Met-CTF was also found to interact with β-catenin in the nucleus of aggressive breast cancer cells [104], suggesting the possibility that Met-CTF promotes β-catenin/Wnt signaling-mediated gene transcription and tumor progression.

Ron, another member of the c-Met subfamily of receptors, translocates from the cell surface into the nucleus of bladder cancer cells upon physiological stress, e.g., serum starvation or oxidative stress [105]. Interestingly, nuclear Ron, in a complex with EGFR, functions as a transcriptional regulator in response to acute disturbances such as serum starvation. ChIP-chip assay indicated that at least 134 genes are potentially targeted by the Ron/EGFR nuclear complex, and motif scanning of the promoters from ChIP-sequence analysis identified a consensus sequence of GCA(G)GGGGCAGCG as the target binding sequence of nuclear Ron upon serum starvation in bladder cancer cells [105]. Moreover, several genes, including c-Jun, FLJ46072, and SSTR1, have been confirmed to contain the consensus binding sequence of Ron and validated as the real targets of nuclear Ron/EGFR complex by ChIP-qPCR [105].

VEGFR Subfamily

Among the members of VEGFR subfamily, only VEGFR2 has been shown to have transactivation function to date. Using immunoprecipitation combined with mass spectrometry, Domingues et al. identified Sp1 as one of the nuclear partners of VEGFR2. The VEGFR2/Sp1 complex binds to Sp1 binding sites in the VEGFR2 promoter to upregulate its expression [106], suggesting a role of VEGFR2 in positive feedback regulation of its own expression.

Ryk Subfamily

Cleavage at the intracellular domain of Ryk by γ-secretase releases its intracellular domain (Ryk-ICD). Ryk-ICD then translocates into the nucleus to function as a nuclear transcriptional cofactor to regulate Wnt target gene expression [107, 108]. In particular, nuclear Ryk-ICD has been shown to promote GABAergic neuron formation and inhibit oligodendrogenesis by transcriptionally regulating key cell-fate determinant genes such as Dlx2 and Oligo2 during ventral embryonic brain development [109].

Ror Subfamily

Ror1, an orphan receptor tyrosine kinase, was recently demonstrated to enter the nucleus of gastric cancer cells transfected with Ror1 plasmid [110]. Nuclear Ror1 could activate several genes involved in regulating actin cytoskeleton, e.g., radixin (RDX), ezrin (EZR), son of sevenless homologue 2 (SOS2), and caldesmon 1 (CALD1) [111]. These results suggest a potential role of nuclear Ror1 in regulating cell migration and cytoskeleton remodeling.

Trk Subfamily

Although the NGF receptor (TrkA) was detected in the nucleus by the early 1980s [38, 39, 112], its genomic functions remain largely unclear. In fact, among the members of the Trk subfamily of RTKs, only TrkA is frequently observed in the nucleus. Nuclear TrkA likely has a role in regulating cell mitosis, because phosphorylated TrkA was found to colocalize with α-tubulin at the mitotic spindle in the glioma cell line U251 upon NGF treatment [113]. In addition, TrkA was reported to interact with the ligand-binding domain of ERβ, and their interaction changed with age in a mouse model, indicating that TrkA may be involved in ERβ-mediated gene expression during aging [114].

8.3.2 Regulation of DNA Synthesis and Replication

Nuclear EGFR has been detected in highly proliferative cells including regenerating liver, uterus of pregnant mouse, and tumor tissues [45, 115], suggesting a role of nuclear EGFR in DNA synthesis and replication. PCNA is an important cofactor of replicative DNA polymerases and an essential component of eukaryotic chromosomal DNA replisome and is involved in DNA replication, DNA repair, and cell cycle control [116, 117]. A study from Wang et al. indicated that nuclear EGFR interacts with and phosphorylates PCNA at the Tyr-211 residue, leading to its protein stability and activity enhancement, which is correlated with an increase of DNA synthesis and tumor cell proliferation [115]. A positive correlation among nuclear EGFR, PCNA, and phospho-Y211 PCNA was observed in a cohort of primary breast tumor samples. In addition, phospho-Y211 PCNA expression was also significantly correlated with poor survival of breast cancer patients [115]. These results are supported by a study in oropharyngeal squamous cell carcinoma in which the expression of PCNA was the only one that correlated positively with nuclear EGFR among the pathological factors tested in anoropharyngeal tumor tissue microarray [118].

8.3.3 Regulation of DNA Repair

DNA-dependent protein kinase catalytic subunit (DNA-PK), a serine/threonine protein kinase, plays a crucial role in regulating the non-homologous end joining (NHEJ) DNA repair process that is activated by DSB. Several lines of evidence suggest an important role of nuclear EGFR in DSB repair. The first clue of the role of nuclear EGFR in DSB-induced NHEJ DNA repair came from the physical interaction between EGFR and DNA-PK in the nucleus, with a consequent increase of DNA-PK activity [119]. Nuclear translocation of EGFR has been linked to an increase of radiation-induced Thr2609 phosphorylation of DNA-PK [119–121], which is essential for the activity and function of DNA-PK in DSB repair and radioresistance. Furthermore,

nuclear EGFR also co-localizes with γ-H2AX loci and associates with γ-H2AX complex upon radiation treatment [122]. In agreement with these findings, NSCLC cells with EGFR somatic mutations (including L858R and ΔE476-E750) which are defective in radiation-induced nuclear translocation failed to activate DNA-PK, and NSCLC cells with expression of these mutant EGFRs were more sensitive to radiation treatment than those with wild-type EGFR [123]. Consistently, EGFR with NLS mutation, which is unable to translocate to the nucleus, has reduced association with DNA-PK, which consequently decreases DNA-PK activity and DSB repair in response to radiation treatment [124]. On the other hand, EGFRvIII (a somatic EGFR mutant without the loss of nuclear translocation ability) can be detected in the nucleus and is able to activate DNA-PK to increase DSB repair [124]. Additionally, nuclear EGFR has been shown to promote DSB repair induced by DNA-alkylating agents (including cisplatin) through its interaction with DNA-PK [124–126].

In addition to irradiation, UV and H_2O_2 are also known to induce DNA damage, and both have been reported to induce EGFR nuclear translocation [127, 128], implying that nuclear EGFR may be also involved in the repair process of other types of DNA damage (e.g., single-strand damage) besides DSB and be a part of the cellular defense system to promote cell survival from lethal stress environments [129, 130]. Moreover, nuclear EGFR has been demonstrated to regulate DNA mismatch repair through activating PCNA [115] that is important for both DNA replication [117] and nucleotide excision repair [131].

ErbB2 is translocated into the nucleus of breast cancer cells upon irradiation and cisplatin treatment, and blockage of ErbB2 by trastuzumab (Herceptin) reduces nuclear translocation of ErbB2, which is accompanied by a decreased cell survival population [132]. In line with this finding, wild-type ErbB2, when translocated to the nucleus, increased DNA repair induced by cisplatin treatment in breast cancer cells, whereas ErbB2-ΔNLS mutant with loss of nuclear translocation ability had no effect on DNA repair. Furthermore, trastuzumab treatment blocked the nuclear ErbB2-stimulated cisplatin-induced DNA damage repair [133]. Taken together, results of these studies suggest that nuclear ErbB2 is important for promoting DSB repair in cancer cells even though its exact mechanism is not yet clear. It should be mentioned that nuclear ErbB2 did not affect the repair of DNA damage induced by etoposide [133], which requires homologous recombination, suggesting the functional specificity of ErbB2 in NHEJ-mediated DSB repair.

Intact ErbB4, rather than cleaved ErbB4-ICD, was recently identified in the nucleus of cardiac myocytes. In contrast to findings for EGFR and ErbB2, treatment with the chemotherapeutic agent doxorubicin reduced the nuclear expression of ErbB4 and increased expression and activation of p53; however, knockdown of ErbB4 in doxorubicin-treated cells surprisingly abolished the activation of p53, indicating that nuclear ErbB4 may be responsible for doxorubicin-induced p53 activation and DSB repair [134]. These findings need to be further validated before a clear conclusion can be drawn.

No information is currently available on the roles of other nuclear RTKs besides the ErbB subfamily receptors in DNA damage repair. Such studies are important and necessary for us to understand the entire role of nuclear RTKs in repairing DNA damage in cancer cells.

8.3.4 Other Functions of Nuclear RTKs

8.3.4.1 rRNA Biogenesis

Within the nucleus, RTKs associate with either chromatin [39, 41, 135, 136] or the nuclear matrix [83, 137, 138]. In addition, RTKs are localized at specific sub-nuclear compartments such as nucleoli [100, 139–141] and nuclear speckles [83, 84] in different type cells. The nucleolus is the site of rRNA biogenesis, including rRNA transcription, rRNA processing and modification, and rRNA assembly and maturation [142, 143]. The nucleolar localization of RTKs indicates that RTKs may have a role in rRNA biogenesis. In supporting this idea, Li et al. recently demonstrated that nuclear ErbB2 interacts with RNA Pol I through β-actin and activates rRNA transcription, with resultant increasing protein synthesis in breast cancer cells [140]. Interestingly, this activation of rRNA expression by ErbB2 seems to be specific for nuclear ErbB2 rather than for cell surface receptor, as blocking traditional downstream pathways of cell surface ErbB2 did not change the rRNA expression and protein synthesis induced by the nuclear receptor. EGFR-associated proteins in the nucleus have been investigated by a proteomic strategy, and some of the proteins determined to associate with EGFR are nucleolar proteins, such as NOL6 (NP_075068), NOC2L (NP_056473), and NOC4L (NP_076983) [53]. Further validation and study is required for the interaction between nuclear EGFR and these nucleolar proteins and their potential roles in rRNA biogenesis. Interestingly, ErbB3 (full length) was found to localize to the nucleolus when cells become polarized and differentiated by growing on permeable filters. Moreover, ErbB3 translocates from the nucleolus to the nucleoplasm and subsequently to the cytoplasm in response to stimulation by its ligand heregulin in human mammary epithelial cells [144], indicating that the nucleolus may be a temporary storage site for cellular ErbB3 to inhibit its cellular function or that ErbB3 in the nucleolus may play a role in regulating nucleolar structure or rRNA biogenesis under specific conditions, e.g., polarization and differentiation. Indeed, a recent report indicated that $p14^{ARF}$, a tumor suppressor, interacts and sequesters ErbB3-80 kDa in the nucleolus to inhibit its target gene transcription by prevent ErbB3-80 kDa from its binding to the gene promoter [68].

8.3.4.2 Nuclear Calcium Signaling

Both IR and c-Met have been found to stimulate nuclear calcium signaling through the inositol 1,4,5-triphosphate (InsP3)/Ca^{2+} pathway when they are translocated into the nucleus [145, 146]. Given the important roles of nuclear calcium in the regulation of gene expression [147] and cell proliferation [148], it is plausible that nuclear IR and c-Met also regulate cell growth and proliferation by stimulating the nuclear InsP3/Ca^{2+} pathway in tumor cells. Further studies are needed to validate the roles of IR and c-Met in nuclear calcium signaling in cancer cells.

8.4 Roles of Nuclear RTKs in Therapeutic Resistance of Cancer Cells

Aberrant activation of RTK signaling has been implicated in most human cancers [3, 7], which provides a rationale for anti-cancer drug development. Indeed, RTK has become one of most attractive therapeutic targets for human cancers. Many anti-RTK therapeutic agents have been designed, developed, and used in clinical trials, and some of them have been approved by the FDA in the last 20 years, including anti-RTK antibodies, antagonists of RTK ligands, small molecule inhibitors of RTK kinase activity, and many others [3, 149–152]. Although promising therapeutic effects have been demonstrated in some human tumors, resistance to anti-RTK therapy occurs frequently in other cancers (intrinsic resistance) or in a set of cancers after long-term treatment (acquired resistance) [153–157], which is reminiscent of other cancer treatments such as chemotherapy [158].

It is clear that multiple mechanisms are responsible for cancer cell resistance to these drugs [153, 159]. DNA damage repair and drug efflux are two of the major mechanisms contributing to cancer cell resistance to chemotherapeutic agents. Several nuclear RTKs have been implicated in therapeutic resistance. As mentioned above, nuclear EGFR associates with DNA repair complex and activates DNA-PK to promote the repair of irradiation- or chemo-induced DSB with a subsequent increase in tumor cell survival [119, 122, 129, 160], and nuclear EGFR also directly phosphorylates PCNA to regulate mismatch repair and increase DNA synthesis, leading to cell proliferation [115]. In addition, nuclear EGFR was recently found to induce irradiation resistance through its association with DNA-PK and PNPase to enhance DNA-PK-mediated phosphorylation and inactivation of PNPase [161]. PNPase is a 3'→5' exoribonuclease responsible for c-Myc mRNA degradation. After phosphorylation by DNA-PK upon irradiation, PNPase losses its exoribonuclease activity and is unable to degrade Myc mRNA when translocated to the cytoplasm, leading to an increase of Myc expression and resistance to irradiation-induced apoptosis [161]. It is worth noting that these aforementioned mechanisms are not mutually exclusive. It is possible that they cooperate with each other when cancer cells receive irradiation to confer cell survival and resistance. In addition to irradiation, cisplatin treatment also stimulates nuclear translocation of EGFR, and once in the nucleus, EGFR contributes to tumor cell resistance to cisplatin treatment [124, 125]. By the use of in vitro established drug-resistant stable clones, nuclear EGFR was shown to be responsible for cancer cell resistance to TKI treatment. Nuclear EGFR transcriptionally activates the expression of the chemo-resistance gene BCRP/ABCG2, which in turn targets gefitinib to pump it out of cells, leading to cancer cell resistance to gefitinib treatment [62, 162]. Similarly, treatment of cells with cetuximab induces EGFR nuclear translocation [163], which has been shown to render cancer cells resistant to cetuximab treatment in both in vitro and in vivo models [164]. However, the cetuximab-induced EGFR nuclear translocation requires further validation due to controversial reports in the literature [127, 165], although

different cell systems and experimental conditions may be responsible for the discrepancies between these studies. In support of the role of nuclear EGFR in drug resistance, the expression level of nuclear EGFR has been shown to correlate significantly with poor survival in several human tumors including breast cancer [11, 12], oral squamous carcinomas [11, 13, 14], ovarian cancer [15], and prostate cancer [17].

Repairing the cisplatin-induced DNA damage requires nuclear translocation of intact ErbB2, which leads to cancer cell survival with cisplatin treatment [133]. Phosphorylation of $p34^{Cdc2}$ at Y15 by ErbB2, probably by nuclear ErbB2, inactivates $p34^{Cdc2}$, leading to M phase entry delay and therefore resistance to paclitaxel-induced apoptosis [166]. In addition to intact ErbB2, ErbB2 variant forms (from either alternative initiation of translation, e.g., ErbB2-CTFs/p95HER2 or from proteolytic cleavage, e.g., p95L) have been shown to induce cancer cells to become resistant to ErbB2 target therapy. Scaltriti et al. first identified ErbB2 C-terminal fragments (ErbB2-CTFs/p95HER2; molecular weight: 95 kDa), which are generated by alternative translation initiation from methionines located near the transmembrane domain of full-length molecule [167]. In breast cancer cells, ErbB2-CTFs localize to the nucleus, the cytoplasm, and the plasma membrane, and those with ErbB2-CTF expression are resistant to trastuzumab (a monoclonal antibody of ErbB2) but not to lapatinib (a dual TKI of EGFR and ErbB2) [167]. Consistently, a xenograft tumor model indicated that tumors expressing ErbB2-CTFs are sensitive to lapatinib but resistant to trastuzumab [168]. This finding was further supported by a clinical study in which 46 patients with metastatic breast cancer and treated with trastuzumab were investigated; patients with tumors expressing ErbB2-CTFs were found to have little response to trastuzumab [167]. However, it is not clear whether the observed trastuzumab resistance was caused by nuclear ErbB2-CTFs or cytosol/plasma membrane-ErbB2-CTFs. In contrast to ErbB2-CTFs, p95L, a cleaved form of intact ErbB2 with molecular weight similar to that of ErbB2-CTFs, was recently found to confer resistance to ErbB2-targeted therapy when it is found in the nucleus. Lapatinib induces p95L expression that is likely through proteasome cleavage to stimulate p95L nuclear translocation. Nuclear p95L is activated by phosphorylation, and cells with this lapatinib-induced p95L nuclear expression have been shown to be resistant to both trastuzumab and lapatinib [169]. On the basis of these studies, the expression of ErbB2 variants could be a useful predicator for resistance to ErbB2-targeted therapy in cancer cells.

Activation of IGF1R signaling and altered chromatin status were found to be important for cancer cells to acquire reversible drug tolerance [170]. In line with this idea, a recent study showed that nuclear IGF1R expression is increased in the gefitinib-resistant hepatocellular carcinoma (HCC) cell line Mahlavu, and gefitinib treatment enhances not only the activity of IGF1R signaling but also the nuclear translocation of IGF1R in a dose-dependent manner that is accompanied by elevated expression of CD133 [171], an important liver cancer stem cell marker [172, 173]. These findings indicate that nuclear translocation of IGF1R may contribute to gefitinib resistance in HCC tumor cells by increasing the cancer stem cell population. However, the exact function of nuclear IGF1R in mediating gefitinib resistance in HCC warrants further investigation. Nevertheless, whether nuclear IGF1R

regulates CD133 gene expression and/or other genes involved in increasing the cancer stem cell population and whether nuclear IGF1R, like nuclear EGFR as mentioned above, activates drug-resistance genes such as ATP binding cassette (ABC) transporters to induce drug excretion, are all interesting questions waiting to be addressed to further our understanding of the role of nuclear IGF1R in driving tumor cells to become resistant to therapeutic agents.

8.5 Strategies Targeting Nuclear Functions of RTKs for Cancer Therapy

As mentioned earlier, nuclear translocation of RTKs is involved in tumor cell growth and therapeutic resistance, and their nuclear expression levels are correlated with poor survival in multiple human cancer types. Thus, blocking the nuclear translocation of RTKs or their nuclear functions may provide a new direction to improve therapeutic efficacy or to overcome therapeutic resistance of cancers. Although this has not yet been extensively explored, several in vitro and in vivo animal studies have shed light on the promising prospects and feasibility of this strategy.

8.5.1 Blockage of Nuclear Translocation of RTKs

While the mechanisms of RTKs nuclear translocation have become much clearer with the efforts by several research groups [8–10, 174–176], the trafficking is a complicated process, and more work is needed to completely understand how RTKs travel to the nucleus under certain cellular conditions. On the basis of the identified trafficking mechanism, several studies have demonstrated that blocking the nuclear translocation of EGFR could be a strategy for cancer therapy and overcoming the acquired resistance of cancer cells to therapeutic agents. For example, inhibition of nuclear translocation of EGFR by tyrosine kinase inhibitor (TKI) has been shown to sensitize tumor cells to irradiation [127]. An artificial system utilizing the $EGFR_{\Delta NLS}$ mutant, which lacks nuclear translocation, significantly suppressed tumor cell growth in softer agar [55] and sensitized cancer cells to cisplatin [125], suggesting the feasibility of inhibiting tumor growth or reversing the drug resistance by blocking nuclear EGFR translocation. Indeed, inhibition of nuclear translocation of EGFR and ErbB2 by lapatinib, a dual TKI of EGFR and ErbB2, sensitized xenografted tumors of gastric cancer cells (N87) to chemotherapy [63], at least, in part, by the inhibition of nuclear translocation of EGFR and ErbB2, which led to a reduction of EGFR- and ErbB2-mediated TS expression. Several studies have indicated that the activity of Src family kinases is crucial for nuclear transport of EGFR in response to irradiation [177, 178] and cetuximab [164, 179]. Therefore, inhibition of Src family kinase activity may also block nuclear translocation of EGFR. Indeed, dasatinib, a potent inhibitor of multiple tyrosine kinases (including the Src family kinases) was shown to inhibit both cetuximab- and radiation-induced EGFR nuclear

translocation in HNSCC [180]. Consistently, treatment of cetuximab-resistant cancer cells with dasatinib resensitized the cells to cetuximab [164]. In addition, irradiation combined with the poly(ADP-ribose) polymerase (PARP) inhibitor ABT-888 significantly increased the susceptibility of HNSCC cells to irradiation, which correlated with the reduction of EGFR nuclear translocation [181], suggesting that the outcomes of patients with HNSCC can be improved when patients are treated by a combination of radiotherapy and PARP inhibitor.

8.5.2 Blockage of Nuclear Functions of RTKs

Blocking the nuclear functions of RTKs is another approach for cancer therapy and overcoming the therapeutic resistance of cancer cells. A few studies have shown the feasibility of this promising strategy to reverse the acquired resistance of cancer cells. For example, a second methionine mutation of the EGFR L858R mutant at T790 (T790M) is known to be responsible for the resistance of cancer cells to TKI treatment in NSCLC [182, 183]. In contrast to the single L858R EGFR mutant, this double EGFR mutant (L858R/T790M) has been detected in the nucleus and interacts with myosin II (MYH9) and β-actin in H1975 NSCLC cells, suggesting that its nuclear translocation may contribute to TKI resistance. Indeed, disruption of cytoskeleton integrity caused by the myosin inhibitor blebbistatin reduced the nuclear interaction of EGFR with MYH9 and β-actin, and cells treated with both blebbistatin and the T790M-specific TKI CL-387,785 showed decreased COX2 gene transcription and increased the sensitivity to CL-387,785, leading to enhanced apoptosis [184]. Similarly, multidrug-resistant cells Kbvin10, Kbtax50, and CEM/VBL, which overexpress P-glycoprotein and are resistant to vincristine and doxorubicin, were found to be more sensitive to the bifunctional alkylating derivatives of 3a-azacyclopenta[a]indene (an DNA interstrand crosslinking agent) treatment through a possible mechanism that blocks the Src/nuclear EGFR cascade and subsequently inactivates DNA-PK and reduces DSB repair [126].

8.5.3 Utilization of Nuclear Translocation to Enhance Cellular Radiotoxicity

In contrast to the therapeutic strategies of blocking nuclear translocation and nuclear functions of RTKs, targeted radiotherapy with Auger electrons is another approach that uses RTK-specific ligands conjugated with radiopharmaceuticals to deliver the radiotoxic effects directly into the nucleus, inducing DNA damage through nuclear translocation of the ligand/receptor complex.

Twenty years ago, ^{125}I-labeled growth factor peptide was used to investigate the nuclear translocation of receptor ligands after binding to their specific cell surface receptors [42, 43], and this mechanism has now been extended to target radiotherapy

for tumors with EGFR overexpression. Reilly et al. first tested the hypothesis that EGF (after binding to its cell surface receptor) is subjected to internalization followed by nuclear translocation, which would deliver the Auger electron emitter ^{111}In into the nucleus of EGFR-positive breast cancer cells for targeted radiotherapy. They found that after internalization and nuclear translocation, ^{111}In-DTPA-hEGF associates with chromatin to induce DNA damage and subsequently reduce cell survival [185]. Moreover, this radiotoxic effects highly selective in human breast cancer cells and shows a good correlation between EGFR expression and ^{111}In-DTPA-hEGF uptake,^{111}In-DTPA-hEGF nuclear localization, DNA damage, and cell-killing activity, which was consistently demonstrated in a panel of breast cancer cell lines, including MDA-MB-468 cells (1.3×10^6 EGFR per cell), MDA-MB-231 cells (2×10^5 EGFR per cell), and MCF7 cells (1.5×10^4 EGFR per cell) [185–187]. In addition, ^{111}In-DTPA-hEGF was shown to effectively inhibit tumor growth of MDA-MB-468 xenografts in athymic mice, with little toxicity to normal tissues [187, 188]. Furthermore, the combination of EGFR TKI (e.g., gefitinib) with ^{111}In-DTPA-hEGF significantly increased the cytotoxic effects of ^{111}In [189], implying the feasibility of combination therapy. Indeed, the combination of ^{111}In-DTPA-hEGF with either anti-ErbB2 antibody (trastuzumab) or with farnesyltransferase inhibitor (L-778,123) increased the nuclear uptake of ^{111}In-DTPA-hEGF in breast cancer cells. Co-treatment of mice bearing 231-H2N (MDA-MB-231 ErbB2-stable transfectants) xenografts with ^{111}In-DTPA-hEGF and L-788,123 significantly prolonged animal survival [190]. Moreover, systemic administration of ^{111}In-DTPA-hEGF in preclinical studies demonstrated that it has no major toxicity to normal tissues and is safe to animals [191], suggesting good potential for further clinical trials.

It is worth noting that EGFR antibody nimotuzumab labeled with Auger electron emitter ^{111}In has also been shown to have a potential for killing EGFR positive and transtuzumab-resistant breast cancer cells recently [192]. Given that EGFR antibody cetuximab is known to induce EGFR nuclear translocation, which leads to the therapeutic resistance of cancer cells [163, 164], it is possible that a combinational treatment consisting of unlabeled normal antibody and ^{111}In-labeled antibody may be a good strategy to overcome tumor cell resistance to EGFR family antibody treatment.

8.6 Conclusion

Given the importance of RTK signaling in regulating crucial cellular processes and the fact that RTKs are considered to be temporally and spatially regulated, the spatial deregulation of RTKs is likely to contribute totumorigenesis and tumor progression and may further affect the sensitivity and resistance of cancer cells to therapeutic agents [7, 193]. In contrast to the canonical signaling cascades of cell surface RTKs, which have been extensively investigated and well documented in the last few decades, the nuclear translocation and nuclear functions of RTKs are still far from clear. Despite much initial skepticism, the concept of nuclear localization and

nuclear functions of RTKs, especially intact RTKs, has now been demonstrated and supported by mounting evidence from numerous investigators and disciplines, and it is being gradually recognized due to the substantial number of research articles published since 2001.

Although the presence of nuclear RTKs might not be absolutely essential for cell growth, the fact that RTKs can translocate into the nucleus under stimulation by their ligands [10] or under certain stress conditions (e.g., liver regeneration, UV, heat, H_2O_2 in the case of EGFR [119, 128]) indicates that nuclear translocation of RTKs may provide cells with some growth and survival advantages. Indeed, one of the major functions of nuclear RTKs identified to date is to regulate the expression of genes involved in cell growth and proliferation, as demonstrated by different research groups using multiple cell types. However, the full consequences of nuclear translocation of RTKs for cancer development remain unclear. For example: (1) Do the nuclear RTKs directly contribute to tumorigenesis? (2) How do cells regulate the nuclear functions and canonical functions of RTKs during tumor development if any differences exist between these two signaling cascades? (3) Is there any cross-talk among nuclear RTKs similar to what is well documented in the literature for cell surface RTKs? (4) Multiple types of nuclear RTKs such as full-length receptors, receptor variants, and receptor fragments have been found in the nucleus (see Table 8.2). Do they share common nuclear targets and/or nuclear functions? (5) How do we develop therapeutic strategies to efficiently target the different locations and diverse functions of RTK in human cancers?

To answer such questions, a good transgenic animal model with RTKs lacking their nuclear translocation ability but without affecting their other functional activities will be helpful in clarifying the exact roles of nuclear RTKs in tumorigenesis. Another approach to understand the nuclear functions of RTKs is to use non-biased approaches to identify their nuclear interacting proteins and transcriptional targets. Currently, most of the functions and/or targets of nuclear RTK identified to date could be explained by the functions of its canonical signaling cascades, which is not sufficient to address the importance of nuclear RTK in cancer development. More in-depth investigation of nuclear RTKs will help us understand the need for RTKs to translocate into the nucleus if they cannot execute their functions at the cell surface. Finally, such newly gained information will provide substantial rationale for us to develop new effective targeted therapies for RTKs in human cancers and other diseases caused by RTK deregulation.

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Chapter 9
Nucleolar Signaling Determines Cell Fate: The RP-Mdm2-p53 Axis Fine-Tunes Cellular Homeostasis

Yong Liu and Yanping Zhang

Abstract One of the main functions of the nucleolus is to conduct ribosome biogenesis, which is the most energy-consuming process in growing cells. Not surprisingly, this process is highly regulated during cellular proliferation when energy consumption levels are relatively high. Various stresses such as genotoxic, osmotic and oncogenic stress as well as metabolic fluctuations converge at the nucleolus and impinge on ribosome biogenesis. Depending on the severity of the insult, nucleolar stress signaling can induce cell cycle arrest, apoptosis or metabolic adaptation. Although mechanisms associated with the nucleolar stress response are complex and remain to be fully elucidated, many of the pathways that convert stress signals into a cellular response link the nucleolus to the tumor suppressor and guardian of the genome known as p53. It is now known that the activation of p53 upon nucleolar signaling occurs predominantly through the ribosomal protein (RP)-Mdm2-p53 axis.

Keywords Ribosome protein • Ribosome biosynthesis • Mdm2 • p53 • c-Myc • Cancer • Metabolism

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9.1 Ribosome Biogenesis

The nucleolus is a non-membrane-bound subnuclear structure found in eukaryotic cells. The nucleolus serves as the cellular location for the assembly of small and large ribosomal subunits, which is crucial to ensure adequate protein synthesis and to maintain cellular homeostasis. The entire process of ribosome biogenesis has been estimated to occupy nearly 60 % of the cellular resources [1]. Ribosome biogenesis consists of three events, which occur in different subregions of the nucleolus: rRNA transcription, rRNA processing and ribosome assembly. Based on the morphology and composition as observed by electron microscopy (EM), the nucleolus can be divided into three functional compartments: the fibrillar center (FC), the dense fibrillar component (DFC) and the granular component (GC) (Fig. 9.1) [2].

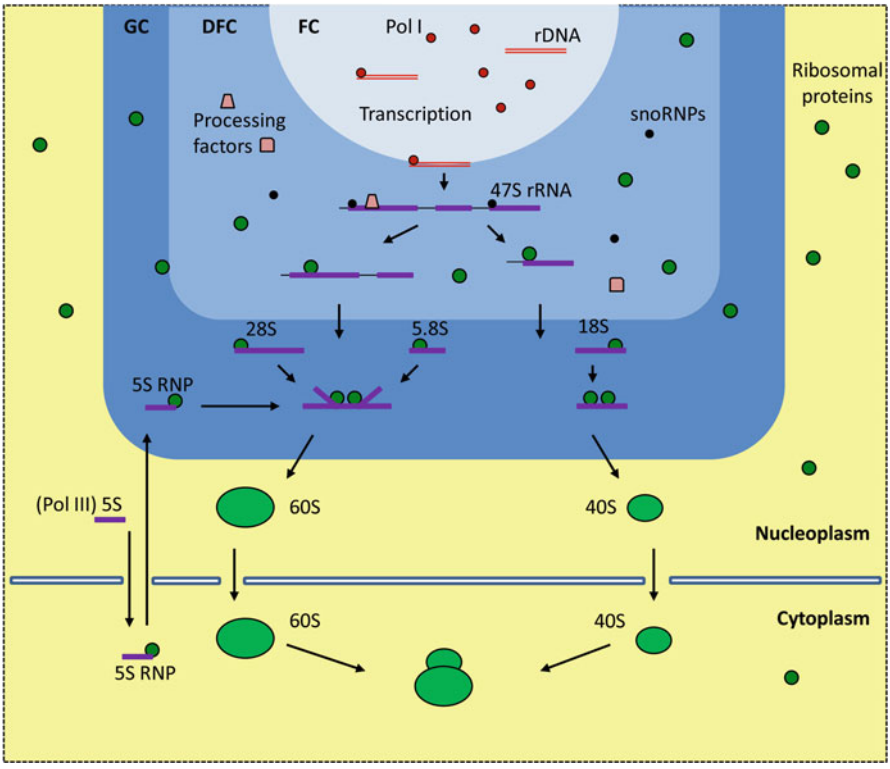

Fig. 9.1 Ribosome biogenesis in the nucleolus. RNA Pol I catalyzes the initial transcription of clusters of rDNA tandem repeat genes into the 47S precursor rRNA either in the FC or at the border between the FC and the DFC. After transcription, the nascent precursors are post-transcriptionally modified through their interaction with snoRNPs and additional processing factors, and the nascent precursors are subsequently processed to form the 5.8S, 18S and 28S mature rRNAs in the DFC. 5S rRNA is transcribed separately by RNA polymerase III (Pol III) in the nucleus, exported to the cytoplasm where it binds to the ribosomal protein L5 to form a complex (5S RNP) and then imported into the nucleolus for incorporation into the large subunit. Once processed or modified, the rRNAs enter the GC region for final maturation and then assemble with many additional ribosomal proteins before becoming transported to the cytoplasm to facilitate protein synthesis

The FC is enriched in components of the RNA polymerase I (PolI) machinery, whereas the DFC harbors rRNA processing factors such as the small nucleolar ribonucleic acids (snoRNAs) and the small nucleolar ribonucleoproteins (snRNPs). The FC and the DFC are enclosed by the GC region, where the final steps of ribosome subunit assembly are conducted.

Based on current evidence, ribosome biogenesis is thought to require the production of roughly equimolar amounts of rRNAs and ribosomal proteins (RPs) to generate 60S and 40S subunits, which mature into 80S polysomes. This process requires the coordinated regulation of all three nuclear RNA polymerases. In humans, RNA PolI catalyzes the initial transcription of clusters of rDNA tandem repeat genes into the 47S precursor rRNA either in the FC or at the border between the FC and the DFC. After transcription, the nascent precursors are post-transcriptionally modified through interactions with snoRNPs and additional processing factors, and the rRNA transcripts are subsequently processed to form the 5.8S, 18S and 28S mature rRNAs in the DFC. In contrast, the smallest component of the large ribosomal subunit, 5S rRNA is transcribed separately by RNA polymerase III (PolIII) in the nucleus. After transcription, 5S rRNA is exported to the cytoplasm where it binds to the ribosomal protein L5 to form a complex (5S RNP), after which the 5S RNP is imported into the nucleolus to be incorporated into the large ribosomal subunit [3]. After processing and modification, the rRNAs enter the GC region where they mature and become assembled along with several ribosomal proteins before being transported to the cytoplasm to conduct protein synthesis. In eukaryotes, 79 ribosomal proteins have been identified, 33 of which are incorporated into the 40S subunit and 46 of which are incorporated into the 60S subunit. All of the genes that encode the ribosomal proteins are exclusively transcribed by RNA PolII [2, 4].

Aside from the ribosomal proteins that are incorporated into the ribosomal subunits, our knowledge on the protein content of the nucleolus remains quite limited. In the past 10 years, proteomic analyses have identified more than 6,000 human proteins that stably co-purify with isolated nucleoli; however, less than 10 % of these proteins have been functionally characterized [5–8]. Among the nucleolus-associated proteins that have been characterized, approximately 30 % directly assist with the production of ribosomal subunits; however, the functions of the remaining 70 % of nucleolar proteins fall into several groups including RNA processing, DNA replication and repair, cell cycle control and apoptosis [2]. Interestingly, additional studies have indicated that the protein content of the nucleolus is dynamic and can be altered in response to various stress stimuli suggesting a complex reorganization of the nucleolus during stress responses [9–11].

9.2 Ribosome Biogenesis and Cancer

9.2.1 Oncogene- and Tumor Suppressor-Dependent Regulation of Ribosome Biogenesis

Based on the fact that ribosome biogenesis is a highly complex and energy-demanding process, one may predict that this process is under constant surveillance

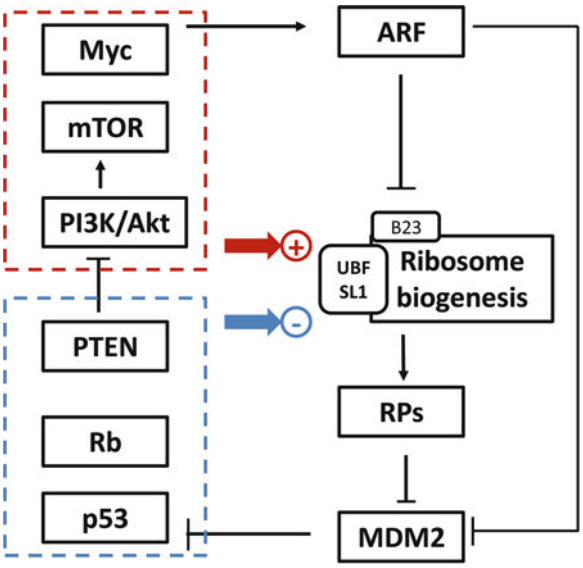

Fig. 9.2 Crosstalk between oncogenes and tumor suppressor genes on ribosome biogenesis. The oncogene Myc promotes Pol I-dependent rDNA transcription by directly binding to SL1 or UBF. At the same time, Myc activates Pol II and promotes RP translation. The PI3K/Akt pathway regulates ribosome biogenesis either through the phosphorylation of S6K to promote 5′-TOP gene expression (RPs) or by promoting rRNA processing and maturation. mTOR promotes rRNA synthesis by activating UBF. Akt also contributes to myc-mediated ribosome biogenesis, and PI3K/Akt signaling is antagonized by PTEN. Oncogenic expression of myc causes the induction of both ARF and RPs. ARF not only directly binds to B23 and inhibits ribosome biogenesis but also interacts with Mdm2 and inhibits Mdm2 E3 ligase activity resulting in the stabilization of p53.p53 interferes with the assembly of the UBF-SL1-Pol I complex and directly inhibits rRNA synthesis. Rb can accumulate in the nucleolus and inhibit ribosome biogenesis by binding to UBF. In a negative feedback loop, myc-induced RP expression results in the inhibition of Mdm2 and further stabilizes p53

at multiple levels [2]. Indeed, the cell must maintain a delicate balance between cell cycle progression and ribosome production, and several key checkpoints exist to assist the cell in keeping this balance. Several tumor suppressors and proto-oncogenes such as p53, Mdm2, ARF, Rb and Myc are sequestered in the nucleoli and regulate aberrant cell division by altering the protein synthesis machinery and coordinating other non-nucleolar signaling pathways such as the PI3K-Akt-mTORC1 signaling axis (Fig. 9.2).

9.2.1.1 Oncogenes and Ribosome Biogenesis

Myc, PI3K-Akt-mTORC1 and Ras-ERK are the three major signaling networks known to promote cell growth when activated, and all of these pathways can become oncogenic if aberrantly activated suggesting a tight correlation between the deregulation of protein biosynthesis and malignant transformation.

Several studies have suggested that Myc regulates ribosome biogenesis, total protein synthesis and cell growth. Overexpression of Myc in normal cells increases the size of the nucleus, the nucleolus and the cell overall, which is associated with cellular proliferation and self-renewal and is consistent with the observation that Myc over expression leads to tumorigenesis [12–14]. Conversely, reduced Myc levels in cancer cells decreases the overall cellular transcription level and may lead to differentiation [15, 16], senescence [17] or apoptosis [18] and a more benign phenotype. In vertebrates, Myc increases the expression of rRNA and ribosomal proteins, and some compelling evidence has suggested that Myc also affects ribosome biogenesis by regulating the expression of the auxiliary factors that are required for rRNA processing, ribosome assembly and the maturation of the ribosomal subunits.

Myc regulates transcription through all three RNA polymerases by binding to DNA as a heterodimer with Max and enabling the recognition of the E-box sequence CACGTG [19]. Grandori et al. have found that when associated with the Pol I-specific factor SL1 (TIF-IB), Myc localizes to the nucleoli at sites of active rDNA transcription and enhances Pol I-dependent gene transcription [20]. Myc also facilitates rDNA transcription by enhancing the expression and the recruitment of the Pol I cofactor known as the upstream binding transcription factor (UBF). SL1 and UBF are indispensible for the augmentation of Pol I-dependent transcription. Myc also directly binds to and activates TFIIIB and augments Pol III-mediated 5S and tRNA transcription [21]. Additionally, Myc activates Pol II-dependent transcription of a large number of genes that encode ribosomal proteins, ribosome assembly proteins and proteins involved in translation initiation and elongation [22–27]. These auxiliary factors include nucleolar protein 56 (NOP56), fibrillarin (FBL), dyskerin (DKC1), block of proliferation 1(BOP1), nucleolin (NCL) and B23 (NPM1 or nucleophosmin) [28]. Notably, B23 is involved in Myc-induced proliferation and transformation by directly interacting with Myc at target promoters of downstream genes. However, B23 has been shown to play an antagonistic role in proliferation by enhancing the stability and transactivation activity of p53, which is important for the maintenance of genomic integrity [29].

The ability of Myc to initiate and promote tumorigenesis is associated with its role in enhancing ribosome biogenesis, and this relationship is particularly evident with the role of Myc in the expression of the ribosomal genes [30, 31]. Several ribosomal proteins such as L19 [32], L7a, L37 [33], L15 [34] and L36a [35] are highly expressed in many different cancers. In addition, the deletion of one allele of L24 or L38 can significantly decrease the incidence of Eμ-Myc-induced lymphoma, which results in a significant delay in tumor onset [31], indicating that even a modest reduction of a single ribosomal protein may attenuate Myc-induced tumorigenesis. However, whether ribosomal proteins exclusively contribute to the transformation of cancer will require further investigation. Interestingly, ribosomal protein L11 has been shown to be a negative feedback regulator of Myc through multiple mechanisms. L11 can directly interact with Myc at the promoters of target genes reducing histone acetylation and consequently inhibiting the translation of Myc target genes [36]. L11 may also inhibit the transcriptional activity of Myc by sequestering Myc to the nucleolus while concomitantly reducing Myc transcript levels. Similarly, L5,

L23 and S7 also bind to Myc, although the functional consequences of these interactions have not been fully elucidated. Interestingly, the ribosomal proteins that bind to Myc have also been shown to interact with Mdm2 to promote p53 stability and activity. Taken together, ribosomal proteins appear to play a dual role in regulating cell proliferation and genomic instability [37, 38].

The PI3K-Akt-mTORC1 pathway regulates the multistep process of mRNA translation at every stage from ribosome biogenesis to translation initiation and elongation. The direct activation of mTOR (mammalian target of rapamycin) by Akt results in the activation of S6 kinase (S6K), which leads to the phosphorylation of ribosomal protein S6 [39]. S6 phosphorylation has been associated with the translation of 5′-TOP genes (terminal oligopyrimidine or tract of oligopyrimidine genes), which have been shown to predominantly encode RP genes, translation initiation factors and translation elongation factors [40].In addition, mTORC1 components RAPTOR (regulatory associated protein of mTOR)and mTOR are both present in the nucleolus. Through the function of mTOR, mTORC1 not only positively regulates the transcription of rRNA and the synthesis of ribosomal proteins, but it is also involved in the processing and maturation of rRNA molecules [41]. mTOR promotes rRNA synthesis through the activation of either transcription initiation factor IA (TIF-IA) or UBF [42]. A recent study also showed that continuous Akt-dependent, mTORC1-independent signaling is required for rRNA transcription elongation. Furthermore, Akt activity is required for the maximal activation of Myc-induced rRNA synthesis in lymphomas, and decreased Akt-dependent rRNA transcription is associated with enhanced apoptosis in lymphoma cells. Collectively, these studies define a network involving Akt, mTORC1 and Myc as master controllers of cell growth and transformation [43]. Additionally, Ras-Erk signaling intersects with both Myc and Akt pathways to regulate each other to promote cell survival [44, 45]. The Ras-Erk-RSKs (p90 ribosomal S6 kinase) axis has also been shown to phosphorylate S6 and to regulate the assembly of the translation preinitiation complex independent of mTOR [46].

9.2.1.2 Tumor Suppressors Involved in Ribosome Biogenesis

Presumably due to the central role of ribosome biogenesis in cell growth, proliferation and tumorigenic transformation, several tumor suppressors such as Rb, p53 and PTEN closely monitor the fidelity of ribosome biogenesis. The tumor suppressor PTEN is mutated in many different types of tumors. Mice that are heterozygous for the PTEN gene are prone to developing tumors of different histological origins [47, 48]. Loss of PTEN function correlates with increases in Akt and S6K activity in tumors. PTEN acts as a phosphatase and is responsible for the dephosphorylation of phosphatidylinositol-3,4,5-triphosphate, which consequently results in the down regulation of the PI3K-Akt pathway. Through the inhibition of the PI3K-Akt-mTORC1 axis, PTEN inactivates S6K and inhibits ribosome biogenesis. Another tumor suppressor known to regulate ribosome biogenesis is the retinoblastoma tumor suppressor gene (Rb). Rb regulates cell cycle progression by directly

inhibiting the transcriptional activity of E2F, which is necessary for S phase progression [49]. Rb has also been shown to modulate ribosome biogenesis. In differentiated cells, Rb accumulates in the nucleolus, and nonphosphorylated active Rb inhibits rRNA synthesis by directly binding to UBF and down regulating Pol I activity [50, 51]. Similarly to Rb, p53 has been shown to repress Pol I transcription by directly interfering with the assembly of the UBF-SL1-Pol I complex [52, 53].In addition to Pol I inhibition, p53 has also been shown to inhibit Pol III activity by binding to TFIIIB thereby compromising the function of TFIIIB in 5S rRNA and tRNA synthesis [54].In addition to modulating ribosome biogenesis, p53 plays a pivotal role in safeguarding deregulated ribosome biogenesis, and the mechanisms through which this occurs will be further discussed below.

9.2.2 Ribosomopathies and Their Associations with Cancer

Almost all ribosome biogenesis factors including the ribosomal proteins themselves are essential for cell survival. Deficiencies in ribosome biogenesis interfere with developmental processes and cause genetic abnormalities, which eventually lead to a collection of human pathological conditions known as ribosomopathies. Over the past few decades, a growing variety of ribosomopathies have been reported, and nearly all of them appear to be associated with an increased incidence of cancer, although the type and frequency vary considerably.

9.2.2.1 Ribosomopathies of Ribosomal Proteins

The first characterized ribosomopathy is known as Diamond Blackfan anemia (DBA) and was originally described and categorized as a congenital hypoplastic anemia in the 1930s [55, 56]. DBA is associated with anemia, macrocytosis, reticulocytopenia and a selective decrease or absence of erythroid precursors in an otherwise normocellular bone marrow [57, 58]. In 1999, recurrent mutations were discovered in the ribosomal protein gene *RPS19* in patients with DBA suggesting the existence an association between mutations in genes that encode ribosomal proteins and DBA [59]. Subsequently, mutations in several other ribosomal proteins including RPS24, RPS17, RPL35A, RPS7, RPS15, RPS27A, RPL36, RPL5 and RPL11 have been identified in approximately 50 % of DBA patients [60–63]. Additional studies revealed that mutations in different ribosomal genes lead to distinct clinical phenotypes by impairing pre-rRNA processing and inhibiting the production of the corresponding ribosomal subunit [64, 65]. The predisposition of DBA patients to cancer remains unclear. Among the 568 DBA patients registered in Diamond-Blackfan Anemia Registry of North America, 15 cancers have been identified in 13 of these patients, which includes four patients with myelodysplastic syndrome (MDS)/acute myeloid leukemia (AML), three patients with osteosarcomas, two patients with colon cancer, two patients with squamous cell carcinomas,

two patients with breast cancers, one patient with a soft tissue sarcoma and one patient with uterine cancer (A. Clachos, Schneider Children's Hospital, Division of Hematology/Oncology, personal written communication, January 2010).

The 5q- syndrome is a subtype of MDS that is preponderant in adult females and is characterized by severe macrocytic anemia, normal/elevated platelet levels with hypolobulated micromegakaryocytes and progression to AML [66]. As implied by the name, 5q- syndrome is characterized by a deletion in the long arm of chromosome 5, which is a critical region containing approximately 40 genes including *RPS14*. RPS14 is a component of the small subunit of the ribosome and RPS14 is required for the processing of 18S rRNA [67]. A recent study that used an RNA interference-based functional screen showed that a partial loss of function of RPS14 phenocopies 5q- syndrome in normal hematopoietic progenitor cells. Furthermore, the forced expression of RPS14 rescues the 5q- syndrome phenotype in patient-derived bone marrow cells harvested from patients with the disease [68]. Notably, both DBA and 5q- syndrome result in a predilection for the development of AML, although the age of onset is quite different between the two diseases.

9.2.2.2 Ribosomopathies of Non-ribosomal Proteins

The small subunit (SSU) processome, composed of the U3 snoRNA and over 40 proteins, is a large ribonucleoprotein (RNP) that is required for the maturation of the 18S rRNA of the SSU. In addition to this complex, many other proteins are required for the maturation of the large and small ribosomal subunits, although most of them are not present in the mature ribosome. Mutations in these ribosome biogenesis factors can act either as a causative agent or as a modifying agent of a particular ribosomopathy such as Shwachman-Diamond syndrome (SDS) and Treacher Collins syndrome (TCS) [57]. SDS is an autosomal recessive disease characterized by exocrine pancreatic insufficiency, ineffective hematopoiesis and an increased risk of leukemia. The *SBDS* gene has been found to be biallelically mutated in approximately 90 % of SDS patients [55]. Although the structure and function of *SBDS*is not fully understood, increasing evidence suggests that this gene plays an important role in ribosome biogenesis and RNA processing [56]. In addition, some ribosomal protein genes such as *RPS9, RPS20, RPL6, RPL15, RPL22, RPL23* and *RPL29* have also shown decreased expression in cells derived from SDS patients suggesting a multiple gene deficiency in SDS. For patients with SDS, a report from the French Severe Chronic Neutropenia Registry estimated the risk of MDS at 19 % at 20 years of age, whereas the risk of AML was estimated at up to 36 % at 30 years of age [58]. TCS is an autosomal dominant craniofacial disorder that includes abnormalities of the eyes, ears and facial bones. The gene *TCOF1* encodes a protein known as treacle and was previously identified as the gene responsible for TCS [69]. Treacle is a nucleolar phosphoprotein that plays a role in rDNA transcription and 18S rRNA gene methylation [70].

Small nucleolar ribonucleoproteins (snoRNPs) are complexes composed of RNA and proteins that localize to the nucleolus. The snoRNPs cause the endonucleolytic

cleavage and chemical modification of pre-rRNA and facilitate the proper folding of pre-rRNA. Mutations in genes encoding either the non-coding RNA components or the protein components of the RNPs tend to lead to ribosomopathies which include cartilage-hair dysplasia (CHH) and dyskeratosiscongenita (DKC) [57]. CHH is an autosomal recessive syndrome characterized by hypoplastic hair, immune dysfunction and a predisposition to various malignancies [71]. The *RMRP* gene encodes a nucleolars no RNA composed of the mitochondrial RNA processing complex (RNase MRP). Mutations within the *RMRP* gene have been suggested to be causative of CHH [72]. In a study of CHH patients with a mean follow-up of 19.2 years, 14 out of 123 patients with CHH were diagnosed with cancer. Of the patients who developed cancer, non-Hodgkin lymphoma was the most common type (nine cases) followed by squamous cell carcinoma, leukemia and Hodgkin lymphoma. In addition, ten patients in CHH cohort also developed basal cell carcinoma of the skin. In general, there was a sevenfold increase in the overall cancer rate in patients with CHH when compared with the normal population [73]. DKC is an X-linked genetic disease characterized by bone marrow failure, mucocutaneous abnormalities and a predisposition to a variety of cancers. In some cases, DKC has also been associated with immune-deficiency, growth retardation and neurological symptoms. DKC can be caused by mutations in different components of the telomerase complex such as telomerase reverse transcriptase (TERT), telomerase RNA (TERC) and dyskerin [74]. A report from the National Cancer Institute DKC cohort revealed an 11-fold increase in the ratio of observed to expected cancers relative to the general population [59].

In addition to these observations in human pathology, in vivo experimental data from animal models also support a model whereby alterations in ribosome biogenesis may promote neoplastic transformation. In *Drosophila*, reduced expression of RPS6 can cause aberrant cell growth and neoplastic transformation in the hematopoietic system [75]. In a zebrafish screen of hundreds of lines of zebrafish with heterozygous embryonic lethal mutations, Amsterdam et al. have shown that 11 of 12 lines with an elevated rate of cancer development harbored a mutation in various ribosomal protein genes [76]. Of note, in these zebrafish lines, the distribution and penetrance of cancers phenocopies the malignancies in p53-null zebrafish. Further analysis revealed that the tumors in zebrafish with ribosomal haploinsufficiency also lost expression of p53 [77]. Moreover, increasing evidence from mouse models further tie together the deficiencies of ribosome biogenesis and tumorigenesis [78–80].

Although each ribosomopathy displays unique characteristics, they share certain features. The most notable similarity between the ribosomopathies is the presence of hypoplastic behavior characterized by decreased cell proliferation and increased apoptosis. In recent years, increasing evidence has demonstrated that the tumor suppressor p53 becomes stabilized and activated in response to impaired ribosome biogenesis. p53 in turn exerts control on cell proliferation and viability providing a link between the activation of p53 and the induction of ribosomopathies [38]. Indeed, evidence is accumulating from studies in animal models that the untimely activation of p53 is responsible for perturbations in tissue homeostasis that cause the development of ribosomopathies such as TCS and 5q-syndrome [81].

9.3 p53 Surveillance of Ribosome Biogenesis

p53 is the principal guardian of genomic stability preventing the initiation and progression of cancer. The tumor suppressor p53 is activated by a broad range of cellular stressors including oncogenic activation, DNA damage, metabolic stress, hypoxia and nucleolar stress [82]. In response to different stresses, p53 transactivates a set of target genes that induces cell cycle arrest, senescence, autophagy and/or apoptosis. Among the growing complexity of roles in cell fate, p53 is known to play a fundamental role in the surveillance of ribosome biogenesis and protein translation. The murine double minute 2 protein (Mdm2, or HDM2 in humans) is the central regulator of p53 that functions as a link between ribosome biogenesis and the p53 pathway. Mdm2 has been shown to bind specifically to several free ribosomal proteins including RPL5, RPL23, RPL11, RPL26, RPS3, RPS7, RPS14, RPS20, RPS25 and RPS27. In an elegant series of experiments, nucleolar disruption, ribosome protein depletion or chemical treatment by agents such as actinomycin D has been shown to cause the release of RPL11 and other ribosomal proteins into the nucleoplasm, which are able to bind to Mdm2 effectively inhibiting Mdm2 activity and eliciting the accumulation of p53 [38, 83].

9.3.1 Function and Regulation of p53

Dubbed the "guardian of the genome" and the "cellular gatekeeper," p53 is the most frequently mutated gene in human cancers, and the mutational spectrum of p53 has been thought to represent a molecular link explaining the etiological causes of cancer. Somatic mutations occur in almost every type of cancer, and even in cancers harboring wild-type p53, the p53 signaling pathway becomes altered [84–86]. p53 functions largely as a transcription factor triggering several different anti-proliferative programs by transcriptionally activating or repressing key effector genes. During the past several years, the p53 transcriptome has emerged as a complex and intriguing field with the discovery of a variety of new and bewildering p53-dependent responses. p53 is responsible for the transcriptional activation or suppression of thousands of genes in stimulus-specific, promoter-dependent and/or cell type-dependent manners. These genes can be functionally categorized into several subsets, which are determined based on the involvement of the particular type of p53 response, including cell cycle arrest, senescence, apoptosis, autophagy and metabolic regulation.

In response to cellular stress, p53 activation consists of three integrated events including p53 stabilization, DNA binding and transcriptional activation of target genes. These processes are regulated by multiple post-translational modifications including phosphorylation, acetylation, methylation, glycosylation, neddylation, sumolyation and poly-ribosylation [87]. p53 stabilization is mainly achieved by disrupting the ability of p53 to interact with Mdm2. As the primary negative regulator of p53, Mdm2-mediated ubiquitination of p53 plays a pivotal role in regulating both p53 turnover and cellular localization. Monoubiquitination of p53 results in the

export of p53 to the cytoplasm, whereas the polyubiquitination of p53 by Mdm2 stimulates the proteasome-mediated degradation of p53 [88]. On one hand, stress signals such as DNA damage cause the activation of various kinases including ATM, ATR, DNA-PK, Chk1 and Chk2, which phosphorylate p53 at specific amino acids. The phosphorylation of p53 at amino-terminal residues such as Ser15 inhibits the interaction between p53 and Mdm2, which results in the stabilization of p53 [89]. Additionally, Mdm2 phosphorylation by ATR has been shown to reduce the Mdm2-dependent exportation of p53 from the nucleus to the cytoplasm and to facilitate p53-induced cell cycle arrest [90]. On the other hand, p53 stabilization can also occur in response to oncogenic challenges to the cell, which is primarily mediated through the antagonism of the p53-Mdm2 interaction by the tumor suppressor ARF [91]. In addition to the phosphorylation of p53, some recent studies have revealed the critical involvement of acetylation events in the selective stimulation of p53-dependent transactivation. Several p53 carboxy-terminal lysine residues are acetylated by CBP/p300, which results in the stabilization and activation of p53 [92], and this mechanism of activation appears to be partially due to the fact that the acetylated lysine residues cannot be ubiquitinated by Mdm2 [93]. Interestingly, lysine 120, which resides within the DNA binding domain of p53, is acetylated by Tip60/hMOF in response to DNA damage resulting in the preferential induction of pro-apoptotic genes instead of those involved in cell cycle arrest, indicating that different modifications of p53 may allow cells to tailor their response to different stress signals [94, 95].

9.3.2 p53 as a Nucleolar Stress Effector Through the RP-Mdm2-p53Axis

9.3.2.1 RP-Mdm2-p53 Axis

As mentioned above, Mdm2 negatively regulates p53 by either conjugating ubiquitin to p53 or by directly concealing the transactivation domain of p53 from the cellular transcriptional machinery [96]. Extensive modifications of Mdm2 through ubiquitination, sumoylation and phosphorylation have also been shown to determine the subcellular localization of Mdm2 and consequently regulate p53 activity [97]. Recently, a subset of ribosomal proteins was shown to inhibit Mdm2 and activate p53 through the extra ribosomal functions of RPs.

The first evidence of RP interaction with Mdm2 involved RPL5 binding to Mdm2 in a 5S rRNA-RPL5-Mdm2-p53 ribonucleoprotein complex [98]. However, the functional consequences of the RP-Mdm2 interaction has not been fully appreciated until recently when several additional studies revealed that other RPs including RPL11, RPL23 and RPL5 could activate p53 through their interaction with Mdm2 [99–103]. Mdm2 is shuttled between the nuclear and cytoplasmic compartments, whereas the RPs are translated in the cytosol and shuttled to the nucleolus to be assembled into the ribosome. Thus, the initiation and maintenance of the interaction between Mdm2 and the RPs requires further investigation. Studies on RPL11

suggested two possible models that may explain the RP-Mdm2 interaction. One model holds that the disruption of the nucleolus promotes the release of RPL11 from the nucleolus to the nucleoplasm, and then RPL11 binds to Mdm2 and stabilizes p53. Another model suggested by Fumagalli et al. proposes that in response to nucleolar stress, cells increase the translation of 5′-TOP mRNAs, which consists of most of the ribosomal proteins including RPL11 [104]. In this model, nucleolar stress causes an increase in RPL11 expression, which increases the concentration of free RPL11 available to bind Mdm2.

Additional evidence for the roles of RPL26 [105], RPS3 [106], RPS7 [107, 108], RPS14 [109], RPS25 [110], RPS27 [111], RPS27L [111–113] and possibly RPS20 [106] as Mdm2 binding partners has also been presented further validating the fact that the Mdm2-p53 interaction is regulated by RPs. Unlike the current understanding of RPL5 and RPL11, some RPs are involved with the Mdm2-p53 interaction in various other mechanisms. RPL26 binds to p53 mRNA and augments the translation of p53, whereas Mdm2 binds to RPL26 and drives the polyubiquitination and proteasomal degradation of RPL26. Moreover, the binding of Mdm2 to RPL26 attenuates the association between RPL26 and p53 mRNA and represses the RPL26-mediated augmentation of p53 gene translation. This balance helps dictate the cellular p53 level and activity in unstressed cells. Genotoxic stress interrupts this balance by enabling a rapid increase in p53 synthesis [105]. Besides RPL26, RPS7 and RPS27L, an RPS27-like protein, are both substrates for Mdm2 E3 ligase activity. Furthermore, RPS25 is a transcriptional target of p53, as p53 directly binds to the RPS25 promoter region and suppresses RPS25 expression. Similarly, RPS27L is a direct p53-inducible target, whereas RPS27 is a p53-repressible target. However, the in vivo significance of the contradictory inter-regulation among RPs, Mdm2 and p53 remains to be determined.

Notably, some recent controversial findings have shown that MdmX (also known as Mdm4) is involved in the regulation of the RP-Mdm2 axis. MdmX, a homologue of Mdm2, is a p53-binding protein. MdmX forms a heterodimer with Mdm2 through their C-terminal RING domains and it greatly increases the capacity of Mdm2 to ubiquitinate and degrade p53 [114]. The activation of p53 by ribosomal stress requires the down regulation of MdmX, and the overexpression of MdmX abrogates p53 activation and prevents growth arrest [115]. In contrast, MdmX facilitates the ability of RPS7 and RPS25 to inhibit Mdm2 E3 ligase activity [108, 110] suggesting a positive feedback loop mediated by MdmX. In addition to MdmX, several novel regulators of the RP-Mdm2 complex have been recently discovered. Promyelocytic leukemia (PML) gene expression was recently shown to enhance p53 stability by sequestering Mdm2 to the nucleolus. Interestingly, the loss of RPL11 expression impairs the ability of PML to localize to the nucleolus and regulate Mdm2. Another RP-Mdm2 binding regulator that has been recently discovered is PICT1 (protein interacting with the C terminus 1, also known as GLTSCR2). PICT1 is a nucleolar protein and has emerged as a key regulator of the nucleolar stress response. PICT1 directly binds to RPL11 and traps L11 in the nucleolus. As a result, PICT1 loss leads to RPL11 release from the nucleolus, inhibition of Mdm2 E3 activity and the accumulation of p53. In human cancers, a lower level of PICT1 expression correlates with a better prognosis, suggesting that PICT1 is a potent regulator of the Mdm2-p53

pathway and promotes tumor progression by retaining RPL11 in the nucleolus [116]. Furthermore, another nucleolar protein MYBBP1A (Myb-binding protein 1a) functions as a link between the cellular energy status and the p53-mediated cell cycle machinery. Upon glucose starvation, eNoSC (energy-dependent nucleolar silencing complex) inhibits rRNA transcription, which results in a reduction in nucleolar RNA content. As a consequence, MYBBP1A, which is anchored to the nucleolus via RNA interactions, translocates from the nucleolus to the nucleoplasm. The translocated MYBBP1A induces the acetylation and accumulation of p53 by enhancing the interaction between p300 and p53 [117]. In addition to RPs, different nucleolar proteins such as B23 [118, 119], PAK1IP1 [120], NEDD8 [121, 122]and several others mentioned above participate in the regulation of p53 stability and activity with or without the involvement of Mdm2 highlighting the importance of the nucleolus in the surveillance of cell proliferation and the determination of cell fate.

Mdm2 contains three conserved regions: an N-terminal p53-binding domain, a central acidic region encompassing a C4 zinc finger and a C-terminal RING domain possessing E3 ligase activity. Previous studies have shown that many cancer-associated alternative and aberrant splicing events in Mdm2 mRNA retain the N-terminal and C-terminal domains but splice out the central acidic domain [123]. In a study involving 23 primary tumors of four types (osteosarcoma, non-Hodgkin's lymphoma, hepatocellular carcinoma and leukemia), eight samples were found to contain mutations in the coding region of MDM2. Notably, most of these mutations target the central zinc finger of MDM2 [124]. Not surprisingly, many RPs including RPL5 and RPL11 form a stable complex with Mdm2 through the direct binding to Mdm2's zinc finger domain [102, 125, 126]. Further analysis demonstrated that the MDM2 C4 zinc finger is critical for the binding of RPL5 and RPL11 to MDM2. A single point mutation converting a cysteine residue to a phenylalanine residue at codon 305 in the zinc finger domain of Mdm2 blocks the binding interaction between Mdm2 and RPL5 and RPL11 but not RPL23[125, 127]. The physiological significance of the RP-Mdm2 interaction was further investigated through the generation of $Mdm2^{C305F}$ knock-in mice [128]. Interestingly, $Mdm2^{C305F}$ mice retain an intact p53 response to DNA damage but fail to stabilize p53 in response to nucleolar stress, which is most likely due to the disruption of the binding between Mdm2 and RPL5 and RPL11. Importantly, the loss of RP-Mdm2 interaction significantly accelerates Eμ-Myc-induced lymphomagenesis, indicating that the RPs-Mdm2-p53 pathway plays a critical role in safeguarding against tumorigenesis. Remarkably, when full-length Mdm2 possessing an acidic domain mutation was used as bait, binding to RPS3 was not affected, suggesting that the interaction between RPS3 and Mdm2 involves more than just the Mdm2 acidic domain [106].

9.3.2.2 Nucleolar Function of p53: Guarding Ribosome Biogenesis

Nucleolar stress specifically refers to perturbations in ribosome biogenesis resulting in the subsequent breakdown of the nucleolar structure. Ribosome biogenesis can be inhibited by serum starvation, contact inhibition, depletion of nucleotides,

treatment with chemotherapeutic compounds such as 5-FU (5-fluorouracil), and the dysfunction of nucleolar proteins involved in ribosome biogenesis. All of these stressors have been shown to generate nucleolar stress that signals to p53 and allows cells to cease proliferation under conditions that sustain low quality or insufficient ribosome biogenesis. According to the disruption that occurs at the different stages of ribosome biogenesis, phenomena that result in nucleolar stress can be placed into three categories: disruption of rRNA synthesis, disruption of rRNA processing and maturation and ribosomal protein imbalance.

The disruption of rRNA synthesis activates p53. As mentioned above, RNA Pol I and Pol III are responsible for the transcription of the rDNA genes. Modifications of RNA Pol I and RNA Pol III have been reported to induce the breakdown of the nucleolar structure and activate a p53 stress response. Several well-established reagent-based experimental systems have been used to mimic the inhibition of precursor rRNA synthesis. Low concentration of actinomycin D (<10nM) specifically disrupts ribosome biogenesis by intercalating into the GC-rich regions of rDNA to inhibit PolI-mediated transcription of nascent 47S rRNA [103, 129].In addition,5-FU, a uracil analog antimetabolite that becomes mis incorporated into nascent RNA and blocks complete RNA synthesis [130],and mycophenolic acid(MPA), an agent that selectively inhibits inosine monophosphate dehydrogenase and depletes the guanine nucleotide pool, which disrupts pre-ribosomal RNA synthesis [131], have also been used to induce nucleolar stress responses to demonstrate p53 stabilization throughRPL5- and RPL11-directed inhibition of Mdm2 [132, 133]. Genetic models such as the deletion of the RNA Pol I transcription cofactor TIF-1A [134], ablation of BAP28 [135] and the inhibition of SL1 recruitment to rDNA promoters by activating PTEN [136]also reduce rRNA synthesis and trigger p53-dependent phenotypes such as apoptosis.

Additionally, the disruption of rRNA processing activates p53. Infidelity in rRNA processing can lead to the accumulation of unprocessed intermediate transcripts, which can retard subunit assembly thereby triggering a nucleolar stress event. One example of this comes from the study of the nucleolar protein Bop1, which is involved in rRNA processing and ribosome assembly. A dominant-negative form of Bop1 (DN-Bop1), which inhibits the transcription of 28S and 5.8S rRNA and causes a deficiency of newly synthesized 60S ribosomal subunits, induces p53-dependent cell cycle arrest in 3T3 fibroblasts. Inactivation of functional p53 reversed the DN-Bop1-induced cell cycle arrest but did not restore normal rRNA processing [137]. Additional study of Wrd36, which is required for 18S rRNA processing, in zebrafish provided in vivo evidence that the disruption of pre-rRNA processing and the inappropriate accumulation of mature rRNA is sufficient to signal a stress response to p53.

Finally, ribosomal protein imbalances can activate p53. A continuous supply of ribosomal proteins is essential for maintaining ribosome biosynthesis and translational machinery. Thus, insufficient RP production can trigger a ribosomal stress response that leads to p53 accumulation and stabilization. Based on in vitro knockdown or in vivo deletion models, the deficiency of several RPs results in the induction of p53-dependent stress responses [138], which indicates that the activation of p53 seems to be a general response of cells to RP insufficiency. However, interestingly,

the phenotypes associated with an RP deficiency are quite heterogeneous. In vertebrates, depending on the specific RP deleted, the resulting effect can either be lethal or pleiotropic manifesting as a specific defect in a particular tissue alongside additional phenotypes, or the deletion of an RP may have no significanteffect. p53 appears to have contrasting roles in determining cell survival or cell death in response to RP insufficiency. Activation of p53 causes embryonic lethality in embryos deficient for RPS6 [139], RPS19 [140] or RPL11 [141]; however, p53 promotes the survival of RPL24-deficient mice during the embryonic stage by delaying the cell cycle and induces a *Bst* phenotype (belly spot and tail) in adults due to apoptosis [142]. Taken together, the RP-Mdm2-p53 axis acts as a checkpoint for ribosome biogenesis and consequently determines cell fate. The stress response could vary between different stages of development and between different tissues in vivo, which is consistent with the differential effects of p53 in different cell types, as p53 is capable of differentiating between cofactors and between different promoters of target genes.

9.3.3 p53 Regulation of Metabolism

As a cellular gatekeeper and a tumor suppressor, p53 plays a major role in sensing and responding to a variety of internal and external stressors to maintain cellular homeostasis. In addition to its classical role in regulating cell cycle arrest, senescence and apoptosis, p53 has recently been shown to regulate metabolism through the transcriptional activation of genes involved in glucose transport, glycolysis, oxidative phosphorylation, glutamine hydrolysis and genes upstream of the mTOR and autophagy pathways.

The primary carbon source for ATP production is glucose. Glycolysis, the enzymatic breakdown of glucose to pyruvate in the cytosol, is an important energy-generating process in cells and is the only alternative to oxidative phosphorylation (OXPHOS) for ATP production. Oxidative phosphorylation is a mitochondrial process in which ADP is phosphorylated to ATP as a direct consequence of oxidizing NADH and FADH2 by the electron transport chain (ETC). Interestingly, in the presence of wild type p53, ATP production is skewed in favor of mitochondrial respiration. However, when p53 is absent, ATP is primarily produced by anaerobic glycolysis [143]. In cancers, tumor cells predominantly metabolize glucose through glycolysis regardless of the surrounding concentration of oxygen (Warburg effect), and this increase in glycolysis correlates with the malignancy of the tumor. Recent findings revealed that p53, which is extensively mutated in cancers, plays a role in governing the switch from aerobic to anaerobic metabolism. The first metabolic gene identified as a p53 transcriptional target is phosphoglycerate mutase (PGM). PGM is a glycolytic enzyme that catalyzes the conversion of 3-phosphoglycerate to 2-phosphoglycerate and drives glycolytic flux [144]. p53 suppress the transcription of PGM and inhibits glycolysis albeit not completely, as PGM is not the rate-limiting enzyme for glycolysis. Later, Vousden et al. identified TIGAR (Tp53-induced glycolysis and apoptosis regulator) as a novel p53-inducible target gene.

TIGAR functions as a potent inhibitor of glycolysis by decreasing the concentration of fructose-2, 6-bisphosphate, which abrogates the activity of phosphofructokinase 1 (PFK1), which is the rate-limiting enzyme for glycolysis [145]. Furthermore, the induction of TIGAR favors the accumulation of fructose-1, 6-bisphosphate, which is effectively shunted into the pentose phosphate pathway to produce NADPH, which acts as a reactive oxygen species (ROS) scavenger and facilitates anabolism. On the other hand, p53 directly regulates OXPHOS by inducing SCO2 (cytochrome c oxidase subunit 2), which regulates the cytochrome *c* oxidase complex of the ETC. Additional studies have shown that the single-allelic deletion of *sco2* is sufficient to recapitulate the glycolytic phenotype observed in p53-null mice, and the expression of SCO2 in p53-null HCT116 cells successfully shifts HCT116 dependence on glycolysis towards the use of OXPHOS substantiating the significance of p53 in the regulation of glucose metabolism [143].

In animals, fatty acids become the main source of energy as blood glucose levels decrease such as when the organism is fasted. Recent studies have begun to unveil the function of p53 in the regulation of lipid homeostasis, which is an important metabolic transition when glucose is not readily available. p53has been shown to inhibit lipogenesis by transcriptionally suppressing SREBP-1c (sterol regulatory element binding protein 1) [146, 147], which is a transcription factor involved in the expression of genes responsible for triglyceride synthesis and lipid accumulation. Furthermore, several genes involved in fatty acid oxidation (FAO) have been characterized as p53 target genes. Upon nutritional stress, p53 becomes phosphorylated at serine residue 18 (equivalent S15 in human), which activates p53 and results in the induction of Lpin1 and the promotion of FAO in mice [148]. p53 has also been demonstrated to promote FAO in response to nutritional stress by inducing GAMT (guanidinoacetate methyltransferase), a critical enzyme involved in creatine biosynthesis and fatty acid oxidation [149]. GAMT catalyzes the conversion of guanidinoacetate to creatine using glycine, arginine or methionine as the substrate. Creatine is produced from these amino acids primarily in the kidneys and the liver, where it is secreted into the peripheral blood circulation for use by myocytes for energy production. Once it is taken up by myocytes, creatine enhances ATP recycling by using a phosphocreatine intermediate to convert ADP to the energy-rich ATP. Under glucose-deprived conditions, GAMT enhances FAO, thereby enhancing this alternative fuel source to maintain constant energy production. The involvement of GAMT in creatine-based energy production indicates that p53 may be involved in energy homeostasis and metabolic shifts between glucose, lipid and amino acid metabolism. In addition to GAMT, other lipid metabolism-associated genes such as CROT (carnitine O-octanoyltransferase), CPT1a (carnitine palmitoyltransferase 1A) [150, 151] and MCD (malonyl CoA decarboxylase) (our unpublished data) have been shown by microarray to be potential p53 transcriptional targets, suggesting that p53 controls lipid metabolism through the mitochondrial and peroxisome oxidation systems.

p53 regulates protein synthesis and degradation mainly through crosstalk with the AMPK and mTOR signaling pathways. As mentioned above, p53 can directly bind to rRNA transcriptional factors and inhibit ribosome biogenesis and protein biosynthesis. Alternatively, p53 transcriptionally up regulates sestrin1 and sestrin2 [152], which are known to protect cells from ROS damage through the regeneration

of overoxidized peroxiredoxins. Sestrin1/2 can also activate AMPK and consequently inhibit mTOR signaling thus promoting the transition from anabolism to catabolism and reserve energy utilization in the interest of cell survival. Interestingly, although the precise signaling pathway remains far from defined, p53 apparently regulates autophagy by two different mechanisms. Nuclear-localized p53 promotes autophagy through the transcriptional activation of the autophagy-inducing protein DRAM (damage-regulated autophagy modulator) and through the inactivation of the mTOR pathway [153–155], whereas cytoplasmic p53 appears to play an opposite role by inhibiting autophagy [152]. Notably, p53-dependent induction of autophagy is considered a stress response. However, at basal levels, p53 regulates autophagy directly at the endoplasmic reticulum (ER). Loss of p53 in the ER causes an ER-specific unfolded protein response (UPR) and results in an increase in autophagy. Interestingly, p53-dependent inhibition of autophagy does not depend on wild-type p53, as several tumor-associated p53 mutants including several gain-of-function mutants can also inhibit autophagy [156]. These observations raise the question of what is the real function of autophagy in the context of tumorigenesis. Clearly, more studies will be required to address this issue.

On a whole cell scale, the cellular redox system is the master regulator responsible for controlling the global cellular metabolism. Reactive oxygen species (ROS) represent an important signaling messenger and stressor. ROS can cause DNA damage and genomic instability leading to the activation of p53 and various stress responses such as apoptosis, cell cycle arrest and senescence. On the other hand, the promotion of OXPHOS is a major metabolic alteration that is brought about by p53 signaling, and interestingly, OXPHOS is the primary intracellular source of ROS. Remarkably, p53 also produces antioxidant products and protects the genome from the oxidation by ROS [157]. Several antioxidants have been reported to be downstream transcriptional targets of p53, including catalase (CAT), manganese superoxide dismutase 2 (MnSOD2), glutathione peroxidase (GPX1), sestrin1/2 and glutaminase (GLS2; responsible for the generation of the major endogenous antioxidant reduced glutathione) [158, 159].As mentioned before, NADPH is also regulated by p53 making p53-dependent antioxidant targets pervasive throughout all cellular redox systems. The involvement of p53 in the production of antioxidants is important in the context of the classical genomic guardian role of p53, as these antioxidants provide potent reducing power to safeguard genomic stability and to prevent senescence and tumorigenesis.

9.4 Conclusions and Perspectives

9.4.1 RP-Mdm2-p53 Pathway Senses Metabolic Alterations and Regulates Metabolic Adaptations

In general, ribosome biogenesis consumes a major portion of the cellular energy supply and resources and plays a key role in the life cycle of the cell. Hence, the cell has developed ways to alter ribosomal biogenesis in the presence of subtle

metabolic fluctuations such as the daily feeding-fasting cycle between meals. Previous studies performed on in vitro cultured cells have shown that metabolic stress such as glucose deprivation can transactivate p53 [117] and induce downstream target genes such as Lpin1 [148] and GAMT [149]. In previous work on the $Mdm2^{C305F}$mouse model, in which the Mdm2 interaction with RPL5 and RPL11 is specifically disrupted, we have shown that this mouse tends to accumulate lipid relative to its wild-type littermate when fed a normal diet. However, when fasted, which indeed inhibits rRNA synthesis, $Mdm2^{C305F}$ mice harbor a phenotype reminiscent of diet-induced obesity, which includes impaired lipid oxidation (our unpublished data). These findings suggest the following: (1) Metabolic fluctuation is a physiological trigger of the RP-Mdm2-p53 signaling pathway; (2) The RP-Mdm2-p53 axis fine-tunes cellular energy homeostasis in low stress or even so-called unstressed conditions.

Other studies have shown that p53 not only performs as a stress-induced player that activates acute responses such as apoptosis but also acts as a housekeeping gene to maintain cellular metabolism within a range that promotes cell survival under low or unstressed conditions. First, p53 functions through stress – and promoter-specific recruitment of transcription initiation. P53 can bind to the promoters of several p53 target genes even in unstressed conditions [160, 161], which suggests that p53 is structurally capable of binding target genes in the absence of stress-induced modifications. Second, previous studies have shown that certain metabolic outcomes of p53 such as the inhibition of autophagy and the induction of antioxidants specifically responds to low or unstressed conditions. Our unpublished data also show that acute stress such as treatment with a low concentration of actinomycin D and other DNA damage inducers only causes the induction of acute response genes such as p21 or Bax, whereas relatively low stress like dietary restriction exclusively induces metabolic target genes. In light of these observations, the physiological function of the RP-Mdm2-p53 pathway appears to involve the detection of metabolic alterations to promote DNA repair (by antioxidants) or to regulate energy homeostasis (by metabolic adaptation) in the interest of cell survival. If this pathway fails to prevent cellular damage, p53-dependent acute responses become initiated by the accumulated stress signals such as DNA damage and ROS resulting in the induction of apoptosis.

9.4.2 RP-Mdm2-p53 Pathway Contributes to the Tumor Suppression Function of p53

Ever since p53 was first categorized as a tumor suppressor, researchers have sought to understand the mechanism of the tumor suppression function of p53. A large amount of findings have shown that acute p53 responses such as cell cycle arrest, senescence and apoptosis could be the key to unlocking several mysteries of cancer treatment, as these processes have been well accepted to be responsible for the success of chemotherapy and radiotherapy. However, several recent studies have raised different issues. By working on an inducible $p53^{ER}$ mouse model combined with

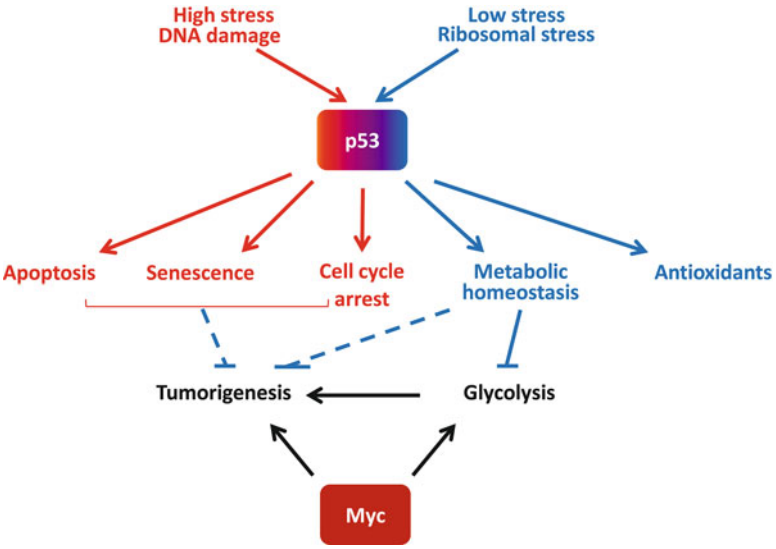

Fig. 9.3 Potential mechanism for p53 to suppress tumorigenesis. *Red color* indicates acute responses induced by intense or accumulated stress; *Blue color* indicates signaling under unstressed or low stress conditions such as metabolic fluctuations. *Dashed lines* indicate signaling that is not well elucidated

irradiation-induced lymphoma, Christophorou et al. have demonstrated that acute radiation responses do not contribute to p53-mediated tumor suppression. Instead, they show that ARF, a tumor suppressor induced by oncogenic expression, plays a key role in p53-mediated tumor suppression [162]. Very recently, another group generated p53 knock-in mice harboring three amino acid substitutions at known acetylation sites ($p53^{3KR}$), which is unable to undergo cell cycle arrest, senescence and apoptosis. Surprisingly, unlike p53-null mice, which succumb to spontaneous thymic lymphomas, early onset tumor formation does not occur in $p53^{3KR}$ mice. Notably, $p53^{3KR}$mice retain the ability to regulate energy metabolism and ROS production, indicating that unconventional activities of p53 such as metabolic regulation and antioxidant function are critical for p53-mediated tumor suppression [163] (Fig. 9.3).

Emerging evidence suggests that metabolic reprogramming is a prerequisite for the rapid cell proliferation of cancer cells. In contrast to differentiated cells, which rely primarily on mitochondrial oxidative phosphorylation to generate the ATP needed for cellular processes, rapidly proliferating cells and cancer cells tend to convert most glucose to lactate through anaerobic glycolysis, which rapidly provides ATP while simultaneously creating the macromolecules necessary for unrestrained biosynthesis typical of cancer cells. As a consequence, RP-Mdm2-p53 axis-mediated metabolic alterations could contribute significantly to tumor suppression. A recent study has provided evidence that nucleolar stress could inhibit tumorigenesis by specifically activating p53 [164]. Verified by both genetic and pharmacological models, this study demonstrated that accelerated rRNA synthesis

and nucleolar integrity are necessary for oncogenic activity. Furthermore, this study showed that the selective inhibition of Pol I is effective in vivo for the treatment of lymphoma and leukemia through the nongenotoxic activation of p53-dependent apoptosis, while sparing normal cells. Combined with our previous findings that inactivation of the RP-Mdm2-p53 pathway increases the incidence of oncogene-induced lymphomas in an ARF-independent fashion [128], the RP-Mdm2-p53 axis conceivably contributes significantly to p53-mediated tumor suppression. However, additional studies are required to address whether metabolic alterations could be the major contribution of the RP-Mdm2-p53 axis to tumor suppression.

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Chapter 10
Transcriptional Regulation of Lipogenesis as a Therapeutic Target for Cancer Treatment

Chenguang Wang, Jun-Yuan Ji, Lifeng Tian, and Richard G. Pestell

Abstract A significant increase in lipogenesis is a metabolic hallmark of proliferating tumor cells and is required for oncogenic transformation of epithelial cells. Although most normal cells acquire the bulk of their fatty acids from the circulation, tumor cells synthesize more than 90 % of required lipids de novo. Consistent with an increased demand for lipid synthesis, diverse human cancer cells express high levels of lipogenic enzymes, such as fatty acid synthase (FASN) and stearoyl-CoA desaturase 1 (SCD1). The sterol regulatory element-binding protein 1 (SREBP1) and peroxisome proliferator-activated receptor γ (PPARγ) are master regulators of lipogenesis in diverse organisms. Previous studies have established that FASN and SCD1, the major transcriptional targets of SREBP1 and PPARγ, promote synthesis of fatty acids, which then serve as ligands for PPARγ activation. This review focuses on the potential therapeutic value of these lipogenic transcription factors as targets in cancer treatment.

Keywords Lipogenesis • Transcription factor • Sterol regulatory element-binding protein 1 (SREBP1) • Peroxisome proliferator-activated receptor gamma (PPARγ) • Cancer • Lipid metabolism • Cell proliferation

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10.1 Introduction

Lipogenesis is almost universally upregulated in human cancers [1]. Consistent with an essential role of the sterol regulatory element-binding protein 1 (SREBP1) in sensing and regulating intracellular lipid homeostasis, increased expression of SREBP1 has been detected in colorectal carcinoma, breast and prostate cancer, and hepatocarcinoma [2–5]. Moreover, elevated expression of SREBP1 is closely correlated with malignant transformation, cancer progression, and metastasis for several cancer types, particularly hormone responsive tissue-derived cancers, such as breast and prostate cancers [2, 4, 6, 7]. SREBP1 expression correlates with the expression of *FASN* (encodes fatty acid synthase or FAS) and Ki-67(a nuclear marker for cellular proliferation) in colorectal cancer, suggesting a role for SREBP1 in supporting rapid cellular proliferation [7]. SREBP1 is elevated in clinical prostate cancer samples compared to benign prostatic hypertrophy [3]. Gene expression profiling of hepatocellular carcinoma (HCC) tissue and non-cancerous liver tissue showed increased lipogenic signaling in HCC. ElevatedSREBP1 expression in hepatocellular carcinoma is a known predictor of increased mortality [4, 6]. Overexpression of SREBP1 in human hepatoma HuH7 and Hep3B cells enhanced cellular proliferation and foci formation, while knockdown of SREBP1 in these cells reduced cell replication and anchorage-independent cell growth [6]. A dramatic increase of SREBP1 has been correlated with the progression of prostate cancer towards androgen-independence [3]. Oncogenic transformation of normal breast epithelial cells was accompanied by increased *SREBP1* and *FASN* expression, consistent with the observation of increased SREBP1 levels in human breast cancers [8–10]. Previous studies have established that SREBP1, through induction of *FASN* and subsequent fatty acids production, regulates PPARγ transactivation [11, 12].

Dietary carbohydrates are digested into glucose, the major source of energy for many tissues. Once transported into cells, glucose is converted into pyruvate through glycolysis and subsequently acetyl Co-enzyme A (acetyl-coA), which is then re-engineered into palmitate, the major fatty acid, by Acetyl-coA carboxylase (ACC), the rate-limiting enzyme, and FAS, the major enzyme, both of which are required for fatty acid biosynthesis. Palmitate is further converted into triglycerides for energy storage and phospholipids, the major components of cell membrane. The key steps in lipogenesis in mammalian hepatocytes are summarized in Fig. 10.1. The enzymatic reactions that govern carbohydrate and lipid metabolism, as well as the allosteric regulation of the activities of these enzymes, also known as the "short-term regulation", have been elucidated by many pioneering biochemists during the first half of the twentieth century.

Compared to the short-term quick regulation of the enzymes, however, the transcriptional regulation of the metabolic enzymes in vivo, known as the "long-term regulation" of metabolism, is less well-understood [13]. Since defects in short-term regulation of enzymes are likely detrimental to survival at the cellular or organismal levels, the aberrant regulation of the long-term regulation contributes to a number of major diseases in adults, collectively known as the metabolic syndrome [14–17].

Because of the fundamental importance of FAS, ACC, ACS and SCD1 in regulating lipid metabolism, it is essential to understand the transcriptional regulation of

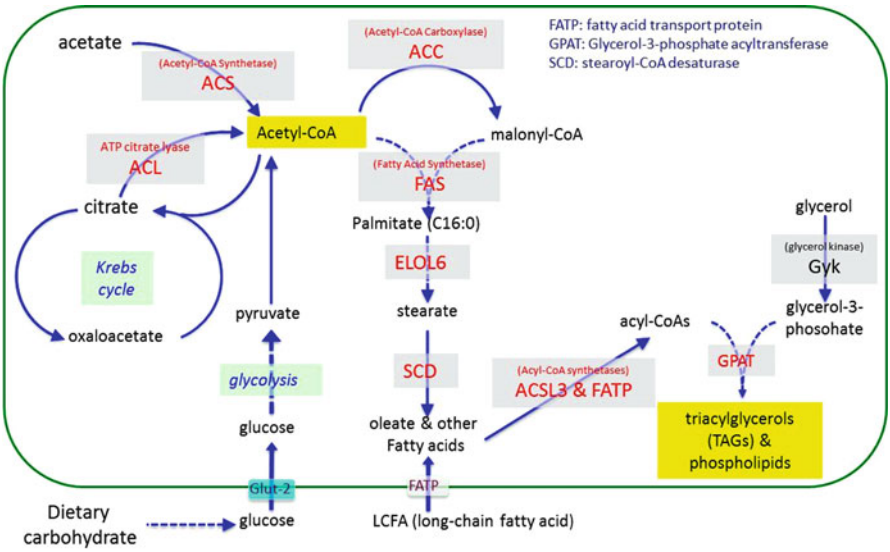

Fig. 10.1 The key biochemical reactions and enzymes involved in de novo lipogenesis in mammalian hepatocytes. This process is highly conserved in evolution. Transcription of many metabolic enzymes in this process is directly regulated by several transcription factors, such as PPARγ, SREBP, ChREBP, and LXR etc.

these enzymes in response to physiological stimuli by key transcription factors, including SREBP, PPARγ, liver X receptor (LXR), and carbohydrate-responsive element-binding protein (ChREBP) [13, 18–20]. In this chapter, we summarize the recent advances in studies linking deregulated lipogenesis in cancers, and then focus on our understanding of SREBPs and PPARγ in regulating lipid homeostasis. Finally, we will discuss potential therapeutic approaches to target lipid metabolism in treating cancer.

10.2 Deregulation of Lipogenic Signaling in Cancer

10.2.1 Elevated FASN Expression and Enhanced De Novo Fatty Acid Synthesis in Cancer

Most normal human tissues preferentially use circulating lipids for synthesis of new structural lipids, and de novo fatty-acid synthesis is normally suppressed due to the low levels of *FASN* expression. In cancer cells, however, fatty-acid supply becomes highly dependent on de novo lipogenesis. Deregulation of de novo fatty-acid synthesis leads to cellular fatty-acid accumulation and affects cellular processes, including signal transduction and gene expression.

FASN over-expression occurs in a variety of human cancers [21–25]. In cancer cells, *FASN* gene expression is upregulated in response to multiple signaling

pathways, including growth factors, steroid hormone receptors such as the estrogen receptor alpha (ERα), androgen receptor (AR) and progesterone receptor (PR), as well as oncoproteins including ErbB2, Ras and Akt [9, 25–30]. In addition to the essential role in cancer cell growth and survival, FAS is involved in other phases of cancer development. FAS over-expression confers resistance to adriamycin and mitoxantrone in breast cancer cells [31] and increased lipogenesis and FAS has been reported to be associated with invasive phenotype and cancer metastasis [24, 27, 32–36]. Elevated expression of *FASN* leads to increased cell proliferation, migration and invasion of prostate cancer cells [27, 32] and FAS inhibition reduces cellular migration and invasiveness [25, 26, 29, 35]. For example, Orlistat, an anti-obesity drug, inhibits FAS function and suppresses endothelial cell proliferation and angiogenesis, suggesting a novel role of FAS in endothelial cell in tumor growth in vivo [37]. It is still unclear how the level and activity of FAS are regulated during tumor progression towards metastasis.

10.2.2 Stearoyl-CoA Desaturase (SCD) and Cancer

SCD is a regulatory enzyme in lipogenesis, catalyzing the rate-limiting step in the de novo synthesis of monounsaturated fatty acids (MUFAs), mainly palmitic and stearic acids. Increased content of the MUFA products, palmitoleic and oleic acids, occurs in a variety of transformed cells and cancers [38–41], suggesting that the high rate of fatty acid synthesis in cancer is coupled to the conversion of saturated fatty acids (SFAs) into MUFAs. Elevated expression and activity of SCD1, the endoplasmic reticulum-resident Δ9 desaturase that converts SFA into MUFA, has been reported in several types of cancers, including colonic and oesophageal carcinoma, liver cancer, and mammary gland tumor [42–45]. SV40-transformed human lung fibroblasts show significantly increased protein and activity levels of SCD1 compared to their parental normal cell line [46]. This is consistent with a model in which a high rate of MUFA synthesis is required for producing membrane lipids in order to sustain the proliferation of transformed cells. Deficiency or inhibition of SCD1 reduces cell proliferation and anchorage-independent growth, and enhances apoptosis in several different cancer cell types [40, 47]. We and others have shown that SCD1 is a transcriptional target of SREBP1 and PPARγ [48–54].

10.3 Cellular Regulation of SREBP1 Function

10.3.1 SREBP1 Signaling in Lipogenesis and Tumorigenesis

SREBPs are a family of transcription factors that control lipid homeostasis by regulating the expression of enzymes required for cholesterol and fatty acids (FAs) synthesis. The three SREBP isoforms, SREBP-1a, SREBP-1c and SREBP-2, have distinct roles in lipid synthesis [55, 56]. In vivo studies using transgenic and

knockout mice suggest that SREBP-1c is involved in FA synthesis and insulin-induced glucose metabolism (particularly in lipogenesis), whereas SREBP-2 is relatively specific in controlling cholesterol synthesis. The SREBP-1a isoform is implicated in regulating both cholesterol and FA pathways [57, 58].

Extensive studies in the past two decades have revealed an elegant paradigm to understand how SREBPs maintain the intracellular lipid and cholesterol homeostasis. SREBP transcription factors are synthesized as inactive precursors bound to the endoplasmic reticulum (ER) membranes and their processing is mainly controlled by cellular sterol content: when sterol level decreases, the precursor undergoes a sequential two-step cleavage process to release the NH2-terminal active domain in the nucleus (designated as the nSREBPs), which then activates SREBP target genes whose products are required for the de novo biosynthesis of cholesterol and FAs [59–66]. The major SREBP targets include FASN [12, 67] and stearoyl-CoA desaturase (SCD) [49–51, 68]. This sterol-sensitive process appears to be a major point of regulation for the SREBP-1a and SREBP-2 isoforms, but not for SREBP-1c. Moreover, the SREBP-1c isoform is mainly regulated at the transcriptional level by insulin. The unique regulation and activation properties of each SREBP isoform facilitate the coordinated regulation of lipid and energy metabolism.

10.3.2 Regulation of the Transcriptional Activity of SREBP1

As summarized above, SREBPs are family of transcription factors that play critical roles in regulating intracellular lipid and cholesterol homeostasis. Using SREBP-1a/-1c as an example, here we focus on the recent advances in our understanding of how SREBP-1 activates lipogenic gene expression and how the transcriptional activity of SREBP is regulated.

10.3.2.1 Transcription Activation by SREBP

In response to cholesterol depletion, the N-terminus of SREBP that contains the transactivation domain and the basic helix-loop-helix leucine zipper (bHLH-Zip) DNA binding domain, is cleaved from its precursor, which is localized in ER and Golgi apparatus, and then translocates to the nucleus and activates the expression of SREBP-target genes [69, 70]. Through the bHLH-Zip DNA-binding domain, the nuclear SREBP fragments bind to the SREBP-target gene promoters that contain either palindromic E-boxes (CAXXTG) or nonpalindromic sterol regulatory elements (SREs) [71].

The transactivation domain of SREBPs can directly interact with transcription coactivators including CBP/p300, PGC-1β, MED14/DRIP150, and MED15/ARC105 [72]. Recruitment of CBP/p300 via the KIX domain of SREBP may alter chromatin structure through the intrinsic histone acetyltransferase activity of CBP/p300, thereby facilitating gene activation [73]. The interaction between PGC-1β and SREBP is required for SREBP-dependent lipogenic gene expression and

contributes to the effect of saturated fat in stimulating hyperlipidemia and atherogenesis [74]. In addition, SREBPs directly interact with the MED14/DRIP150 and the MED15/ARC105 subunits of the Mediator complex in mammals and *C. elegans*, which provides an elegant model to explain how transcription activator SREBPs interact with the general transcription machinery [73, 75–77]. The interactions between SREBP-1c and MED14 or MED15 are weaker than the interactions between SREBP-1a and MED14 or MED15, which may explain why SREBP-1a is more potent than SREBP-1c in activating gene expression [73, 75]. Since the nuclear SREBPs bind to DNA as homo-dimers, it is unclear whether the two transactivation domains of the SREBP homo-dimer can bind to MED14 and MED15 simultaneously.

10.3.2.2 Inactivation of SREBP-Mediated Transcription

Because of the fundamental roles of SREBPs in regulating the expression of lipogenic and cholesterogenic genes, the mechanisms that restrain SREBP transactivation are also important. CDK8, the enzymatic subunit of the Mediator complex, directly phosphorylates a conserved Threonine residue in SREBP (Thr402 in SREBP-1c), thereby promoting nuclear SREBP degradation [78]. Consistent with this model, the mutants of CDK8 and its regulatory partner Cyclin C (CycC) in *Drosophila* larvae, as well as depletion of CDK8 in cultured mammalian cells and mouse liver, display significantly increased expression of SREBP-target genes and dramatic increase of triglyceride accumulation [78]. Feeding and activation of the insulin-signaling pathway can down-regulate CDK8-CycC thus allow the activation of nuclear SRBEP, providing a mechanism for the lipogenic effect of insulin [78]. Together with the previous works on MED14 and MED15 in activating SREBP-dependent gene expression, this recent work on the inhibitory effect of CDK8-CycC on SREBP-regulated de novo lipogenes further highlights the importance of the Mediator complexes in modulating the activation and subsequent degradation of nuclear SREBPs.

Interestingly, GSK3β also negatively regulates SREBP by phosphorylating SREBP-1a at Thr 426 and Ser430 (corresponding to Thr402 and Ser406 in SREBP-1c), thereby providing a docking site for the ubiquitin ligase FBW7 [79–81]. It is still not known whether CDK8 and GSK3β play redundant roles in phosphorylating and thereby promoting SREBP destruction, however, these studies suggest a model to explain how activation of SREBP-dependent transcription is coupled to its degradation. This mechanism is consistent with a general theme by coupling transactivation with their destruction for many transactivators in yeasts and multicellular organisms [82].

Both CDK8 and CycC are amplified, mutated or deleted in a variety of cancers, and CDK8 is identified as an oncoprote in melanoma and colorectal cancers [83]. In addition, the MED12 subunit of the CDK8 module, which is composed of CDK8, CycC, MED12 and MED13, is mutated in prostate cancer, colorectal cancer, and

~70 % of uterine leiomyomas [84–87]. Importantly, MED12, but not MED13, is required for human CDK8 kinase activity [88]. Therefore, although it is still unclear how dysregulation of the CDK8 module contributes to tumorigenesis, it is conceivable that dysregulation of CDK8 submodules may compromise CDK8 activity, thereby potentiating SREBP activity, increasing SREBP target gene expression and promoting lipogenesis in cancer cells. This model may explain the mechanisms underlying aberrantly increased lipogenesis in human cancer cells and provide the rationale for developing pharmaceutical approaches to block de novo lipogenesis in tumor cells.

10.4 Modulation of PPARγ Activation for Cancer Therapeutics

10.4.1 The Function of PPARγ in Lipogenesis

Besides SREBPs, the peroxisome proliferator-activator receptor gamma (PPARγ) also plays a critical role in both lipid metabolism and tumorigenesis. The PPARs are ligand-activated nuclear receptors, which include PPARα, PPARγ and PPAR*δ* [89]. Their modular structure resembles other nuclear hormone receptors with an N-terminal activation function 1 (AF-1), a DNA binding domain, and a C-terminal ligand-binding domain that harbors AF2. PPARγ was initially cloned as a transcription factor involved in adipocyte differentiation. Subsequent studies suggested a broad spectrum of PPARγ functions in lipid metabolism, inflammation, atherogenesis, cell differentiation, as well as tumorigenesis. The endogenous PPARγ ligands include derivatives of fatty acids produced through lipogenesis (Fig. 10.1).

PPARγ regulates lipogenesis and adipocyte differentiation, and ectopic PPARγ expression promotes cell adipogenesis in an NIH 3 T3 cell model [90, 91]. Synthetic PPARγ ligands enhance de novo lipogenesis [92, 93], which was further supported by the genetic evidence that *PPARγ*$^{-/-}$ ES cells and embryonic fibroblasts are resistant to induction of adipogenesis [94, 95]. Aberrant hepatic expression of PPARγ2 stimulates murine hepatic lipogenesis [96, 97]. The screening for PPARγ-regulated genes in mammary epithelial cells, identified that Scd1 (*SCD1*) as a transcriptional target of PPARγ [98]. SCD1 production of unsaturated fatty acids may thereby serve as PPARγ agonist ligands, providing a feedback loop to PPARγ. Reciprocal up-regulation of PPARγ and SREBP-1 has been reported. Ectopic expression of SREBP-1 in pre-adipocyte 3 T3-L1 cells and hepatic cancer HepG2 cells induced endogenous PPARγ mRNA expression [99]. SREBP-1 activation increased the production of lipids as endogenous ligands for PPARγ, which binds to PPARγ and augments the transcriptional activity of PPARγ [11, 12]. PPARγ, upon ligand binding, up-regulates the expression of *INSIG1*, the key regulator in the processing of SREBPs [100].

10.4.2 Contradictory Role of PPARγ in Tumorigenesis

PPARγ has been shown to function either as an oncoprote in, or as a tumor suppressor. PPARγ is expressed in breast, prostate and colonic epithelium and ligand-dependent activation of PPARγ in cell lines derived from these tumors inhibits cellular proliferation [101–105]. Consistent with the role of PPARγ as a tumor suppressor, PPARγ ligand reduced tumorigenesis in the Apc^{Min} model of familial adenomatous polyposis. Carcinogen, N-nitroso-N-methylurea (NMU)-induced mammary tumorigenesis is prevented by PPARγ agonists [106], and 7, 12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumorigenesis was inhibited by troglitazone [107–109]. A chromosomal translocation between PAX8 and PPARγ in follicular thyroid cancer served as a dominant inhibitor of endogenous PPARγ expression [110]. PPARγ expression is reduced in human breast cancers compared with normal breast tissue [111, 112] and PPARγ over expression in tumor cells inhibits cell proliferation in tissue culture [112]. PPARγ levels are reduced in mouse transgenic mammary tumors induced by distinct oncogenes, compared with normal adjacent non tumorous mammary epithelium [111, 112].

In contrast, evidence that PPARγ is an oncogene includes observations that PPARγ ligands promote colonic tumor growth in Apc^{Min} mice when fed a high fat diet [113]. Heterozygous mutations of PPARγ have been detected in 4/55 patients with colon cancer [114]. Although genetic analyses failed to show that PPARγ-deficient mice develop enhanced tumor phenotypes in prostate epithelium induced by the SV40 large-T antigen oncogene [113]. A constitutively active mutant of PPARγ (PγCA) enhanced ErbB2-induced tumor in vivo in immune-competent animals (Fig. 10.2) and in transgenic mice [115]. PγCA promoted ErbB2-induced tumor growth in immune-competent animals. Increased angiogenesis is associated with enhanced tumor growth in vivo [116]. Collectively, these studies suggest cell-type specific functions of PPARγ in the tumor induction versus inhibition.

10.4.3 Can PPARγ Be Targeted to Block the Tumor Growth?

In cell culture, PPARγ expression and/or activation repressed tumor cell growth by inhibiting cell proliferation, promoting apoptotic and autophagic cell death, and inducing terminal differentiation of cancer cells [112, 117, 118]. In whole animal studies, the picture appears more complex, which is consistent with the importance of heterotypic signals in cancer progression, and the presence of PPARγ in a variety of cell types including the inflammatory system. Clinical trials have been undertaken in a variety of tumor types including liposarcomas, prostate, pancreatic, colorectal, breast, thyroid, head and neck cancers, as well as melanoma and leukemia [119]. Overall, PPARγ agonists failed to yield positive clinical outcome in most cancer types. PPARγ is increased in ERα-negative breast cancer, but reduced expression in ERα-positive breast cancers [120]. Breast cancer genetic subtypes

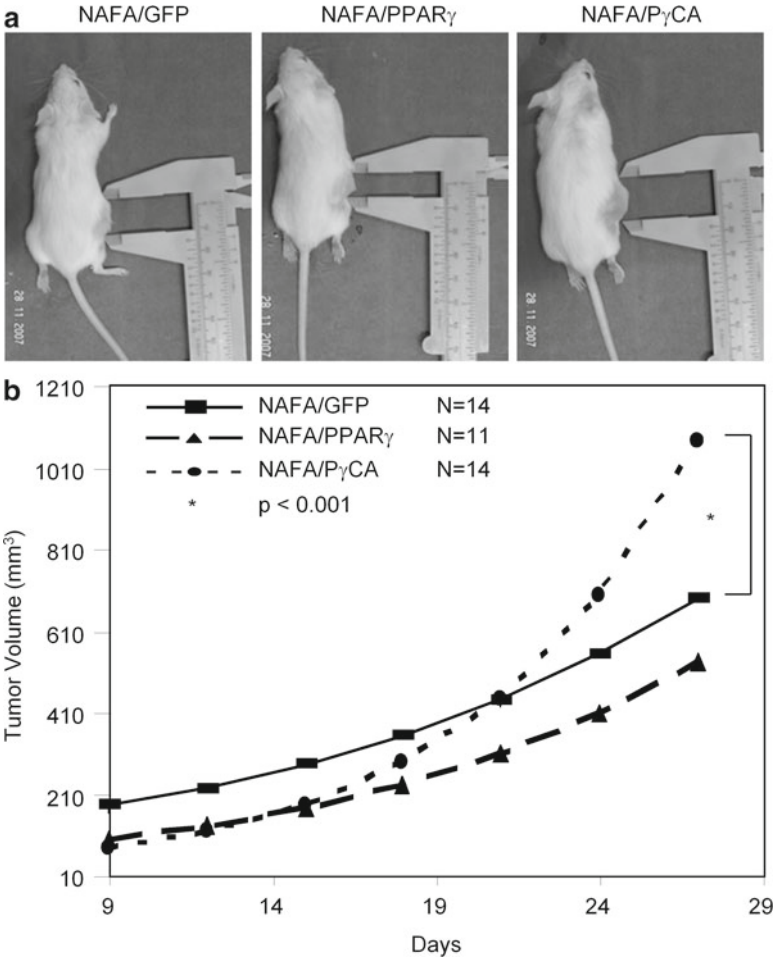

Fig. 10.2 PγCA promotes tumor growth in vivo. (**a**) NAFA cells transduced with MSCV-IRES-GFP vector encoding either PPARγ, PγCA, or empty vector were implanted into FVB by injecting 2×10^6 cells subcutaneously. Tumor growth was measured every 3 days by digital caliper and tumor volume was calculated. (**b**) Tumor volumes were logarithm-transformed and analyzed using a linear mixed model. Separate slope and intercepts were computed for each group (GFP, PPARγ, and PγCA), then compared across groups using a global test followed by pair-wise comparisons via linear contrasts (This figure was reproduced from our previous publication [116])

(Luminal A, Luminal B, Triple negative/basal-like, HER2 subtypes [121–123]) may have different response to PPARγ ligands. Our analysis of *PPARG* gene expression in a combined dataset comprising of over 2,000 breast cancers [124] showed a strong heterogeneous distribution of *PPARG* expression among the subtypes (unpublished data). Consistent with our previous IHC result showing reduced PPARγ expression in breast cancer comparing to normal breast tissue, this analysis demonstrated that the gene expression of *PPARG* was also reduced (data not shown).

The higher *PPARG* expression predicts a better clinical outcome, which again holds the promise that PPARγ could serve as a therapeutic target. Given the variability in PPARγ expression in patient populations, clinical trials using PPARγ expression or function as a companion diagnostic may be warranted.

10.5 Conclusions and Future Directions

Given the importance of lipogenesis in cancer development, targeting lipogenic signaling, particularly lipogenic enzymes, is an attractive strategy. The inhibitors of the rate-limiting or key lipogenic enzymes, including HMGCR, ACC, FASN, and SCD, are summarized in Table 10.1. In addition to inhibiting these lipogenic enzymes, pharmaceutical inhibition of SREBPs and PPARγ, may also be effective.

Taken together, these studies suggest that dysregulated lipogenic signaling in cancer is required for oncogenic transformation, thus targeting the dysregulated lipogenesis in câncer cells may represent an attractive therapeutic approach. Current

Table 10.1 Chemical modulators of lipogenic signaling

Modulator	Targeting molecule/pathway	Mechanism of function	References
Statins	HMG-CoA reductase (HMGCR)/mevalonate pathway	Structural analogs of HMG-CoA reductase, lipid-lowing agent	Review in [125]
Soraphen A	Acetyl CoA carboxylase (ACC)	Interferes with fatty acid elongation	[126, 127]
benzofuranyl alpha-pyrone (TEI-B00422)	Acetyl CoA carboxylase (ACC)	Competitive inhibition of ACC	[128]
5-(tetradecyloxy)-2-furoic acid (TOFA)	Acetyl CoA carboxylase (ACC)	Long chain fatty acid analogues	[129]
CP-640186	Acetyl CoA carboxylase (ACC)	Interacts with ACC	[130]
Cerulenin	Fatty acid synthase (FASN)		[131, 132]
C75	Fatty acid synthase (FASN)	Interacts and inhibits FASN	[133, 134]
C93	Fatty acid synthase (FASN)		[135]
Orlistat	Fatty acid synthase (FASN)		[136]
EGCG	Fatty acid synthase (FASN)		[137]
G28UCM	Fatty acid synthase (FASN)		[138]
GSK837149A	Fatty acid synthase (FASN)	Target the beta-ketoacyl reductase reaction	[139]
MK-8245	Stearoyl-CoA desaturase (SCD)		[140]
Compound 9	Stearoyl-CoA desaturase (SCD)		[141]
Fatostatin (and derivatives)	SREBP-1	Inhibits SREBP-1 processing	[142, 143]
BF175 (and derivatives)	SREBP-1	N.D.	[144]
TZDs	PPARγ	Interacts with and activate PPARγ	[145]

research efforts have been focused on repressing the activity of lipogenic enzymes (such as FASN, HMG-CoA reductase, ACC, ACLY, and SCD). Future studies are required to provide a deeper understanding of the following three major aspects. First, it would be important to understand how alterations in molecular mechanisms of lipogenic signaling occur in cancer. Second, a compendium of metabolic profiling in different cancer types and subtypes may allow for more accurate patient selection for specific lipogenic pathway targeted therapies. Third, it may be important to simultaneously target multiple lipogenic factors rather than a single molecule, to ensure therapy effectiveness.

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Part III
Protein Domains and Cancer Therapeutics

Chapter 11
Selective Inhibition of Acetyl-Lysine Effector Domains of the Bromodomain Family in Oncology

Susanne Müller, Hannah Lingard, and Stefan Knapp

Abstract Acetylation of lysine residues is a posttranslational modification that plays a key role in the regulation of chromatin structure and transcription. In cancer, aberrant lysine acetylation often leads to changes in gene expression resulting in inactivation of tumour suppressor functions and the activation of pro-survival and proliferation promoting pathways. Enzymes that "write" (acetyltransferases, HATs) and "erase" (histone deacetylases, HDACs) ε-N-acetyl-lysine (K_{ac}) marks have therefore emerged as interesting targets for the development of novel drugs for cancer treatment. Recently also acetyl-lysine reader domains have gained interest as novel targets for pharmacological intervention. The acetyl-lysine mark is specifically recognized by the bromodomain family of protein interaction modules. Bromodomains are present in diverse nuclear proteins regulating the recruitment of transcriptional regulators and chromatin modifying enzymes and proteins to acetylated chromatin as well as proteins mediating the assembly of other nuclear protein complexes. Dysfunction of bromodomain containing proteins such as chromosomal rearrangements and aberrant expression of these proteins in cancer has been tightly linked to tumourigenesis. Recently identified inhibitors that selectively target bromodomains demonstrated potent anti-tumour activity, suggesting new avenues for the development of antineoplastic drugs. In this chapter we will review the current knowledge of the role of bromodomains in tumour development and identified selective inhibitors developed to disrupt acetyl-lysine dependent protein interactions mediated by this family of transcriptional regulators.

Keywords Bromodomain • Protein acetylation • Epigenetic reader modules • Transcription • Chromatin • BET • ATAD2 • BRG1 • TIF1alpha • BRD-NUT

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Abbreviations

AML	Acute myeloid leukemia
ASH1L	Absent, small or homeotic-like
ATAD2A/B	AAA domain-containing protein 2
ATP	Adenosine triphosphate
BAZ	Bromodomain adjacent to zinc finger domain protein
BCL2	B-cell lymphoma 2
BET	Bromodomain and extra-terminal
BRD	Bromodomain-containing
BRPF	Bromodomain and PHD finger-containing protein
BRWD	Bromodomain and WD repeat-containing protein
CECR	Cat eye syndrome critical region
CML	Chronic myeloid leukemia
c-MYC	V-myc myelocytomatosis viral oncogene homolog
CREBBP	CREB-binding protein
EP300	Histone acetyltransferase p300
FALZ	Fetal Alzheimer antigene
FOSL1	FOS-like antigen 1
GCN5L2	General control of amino acid synthesis protein 5-like 2
HAT	Histone acetyltransferase
HBO1	HAT bound to Orc1
HCF-1	Host cell factor 1
HDAC	Histone deacetylase
HIV	Human immunodeficiency virus
hMOF	Human ortholog of fly mof, MOF, for males absent on the first
MLL	Mixed Linage Leukemia
MORF	MOZ-related factor
MOZ	Monocytic leukemic zinc finger protein
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMC	NUT midline carcinoma
NSCLC	Non-small cell lung cancer
NUT	Nuclear protein in testis
PB1	Polybromo 1, PBAF, Polybromo and Brg1-associated factor
PCAF	P300/CBP associated factor
PHIP	PH-interacting protein
PRKCBP1	Protein kinase C-binding protein 1
P-TEFb	Positive transcription elongation factor complex (cdk9/cyclinT)
Rara	Retinoic acid receptor alpha
RNA	Ribonucleic acid
siRNA	Small interfering RNA/Short interfering RNA
SIRT	Sirtuin
SMARCA	SWI/SNF-related, matrix-associated actin-dependent regulator of chromatin
SP100/110/140	Nuclear body protein

SWI/SNF	SWItch/Sucrose NonFermentable
TAF1/TAF1L	Transcription initiation TFIID associated factor
Tat	Trans-activator of transcription
TIP60	HIV Tat-interactive protein of 60 kDa
TRIM24/28/33/66	Transcription intermediary factor 1-alpha (TIF1α)
WDR9	Bromodomain and WD repeat-containing protein 1
ZMYND11	Zinc finger MYND domain-containing protein 11

Epigenetics has been defined as heritable regulatory mechanisms regulating gene transcription that are not encoded by the DNA sequence itself. These regulatory mechanisms comprise dynamic changes in chromatin structure mediated by post-translational modifications (PTMs) in proteins that organize chromatin such as histones.

Dense packing of chromatin requires neutralization of the high negative charge density of DNA by basic histones. Acetylation has a profound effect on the physico-chemical properties of the lysine side chain by neutralizing the charge of ε-*N* amine. This property is thought to favour an open, more loosely packed state of chromatin, leading to increased accessibility of regulatory regions on DNA and transcriptional activation. In cancer, inappropriate acetylation levels give rise to aberrant expression of genes that promote tumourigenesis. Both hyper- as well as hypoacetylation in promoter regions have been observed, leading to overexpression of growth and survival promoting genes as well as repression of tumour suppressor genes.

11.1 Role of Acetylation Homeostasis Regulating Transcription

ε-*N* lysine acetylation is one of the most frequently detected PTMs [1]. Acetylation homeostasis is principally controlled by enzymes that "write" acetylation marks (histone acetyltransferases (HATs)) or erase them (histone deacetylases, (HDACs)). Based on their homology with the corresponding yeast proteins the 18 human HDACs have been grouped into four different classes. Class I, II and IV are referred to as classical HDACs. They comprise HDAC1, HDAC2, HDAC3 and HDAC8 (class I), HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10 (class II) and HDAC11 (class IV). These enzymes share a common mechanism of action and harbour a Zn^{2+} ion in their active site. In contrast, class III enzymes require NAD^{+} as cofactor and comprise the sirtuins SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, and SIRT7 [2]. The HDAC subfamilies differ in their tissue specific and subcellular localisation, which has an influence on their substrates and biological function. While class I HDACs are uniformly nuclear, ubiquitously expressed proteins that deacetylate histones, other classes of HDACs like class IIa HDACs are expressed in a tissue specific manner and shuttle between nucleus and cytoplasm [3]. Accordingly, HDACs influence gene transcription on different levels and they have been reported

to regulate chromatin as well as non-chromatin proteins. HDACs can drive carcinogenesis through a variety of different mechanisms: truncating mutations of HDAC2 have been identified in sporadic carcinomas with microsatellite instability and in tumours arising in individuals with hereditary nonpolyposis colorectal cancer syndrome [4], however most commonly dysregulated expression of HDACs has been observed in a wide variety of tumour types [2, 5]. HDAC overexpression can lead to upregulation of genes involved in development and proliferation [6] or downregulation of tumour suppressors [7]. HDACs may also regulate microRNAs (miRs) [8].

A variety of inhibitors against HDACs have been developed and currently two of these, suberoylanilidehydroxamic acid (SAHA, Vorinostat, Zolinza™) and depsipeptide (Romidepsin, Istodax™) have been approved by the US Food and Drug Administration. Most HDAC inhibitors are not target specific and inhibit most frequently class I enzymes. The precise mechanisms of action of HDAC inhibitors are not well understood and inhibition of the enzymatic activity as well as disruption of interactions with other proteins may play a role in the observed phenotypes [5, 9, 10].

Addition of acetyl groups to lysine residues is carried out by HATs which are classified into two different groups, class A HATs, which are nuclear and acetylate histone and non-histone proteins and the less characterised, cytoplasmic class B HATs, which are thought to acetylate newly synthesised histones and play a role in DNA repair and chromatin assembly [11, 12]. Nuclear HATs include 3 families, the GCN5L2/PCAF, the CREBBP/EP300 and the TAF families. In addition, the MYST family consisting of the five human HATs hMOF, TIP60, HBO1 (HAT bound to Orc1, also named MYST2), MOZ, and MORF belong to the class A HAT family [13]. HATs acetylate specific lysine residues on histone tails as well as non-histone proteins. At the chromatin level acetylation is generally thought to be associated with the formation of euchromatin and transcriptional activation. Acetylation of non-histone proteins can result in alteration of DNA binding, protein-protein interactions, protein stability or subcellular localisation [14].

TAF1, TAF1L, PCAF, GCN5L2 and EP300/CREBBP contain bromodomains in addition to the HAT domain, which will be discussed below and which offer alternative sites for pharmacological modulation of HAT function. Contrary to HDAC inhibitors, the development of inhibitors of HATs has not been as successful so far and only few natural products acting with low potency have been reported [15].

Until recently, little attention has been dedicated to inhibitor development and in understanding the function of protein interacting domains that "read" the epigenetic code but recent discoveries of highly potent and specific inhibitors for bromodomains strongly stimulated efforts targeting these domains. The recognition of the acetyl-lysine mark is principally mediated by bromodomains, a family of 61 diverse interaction modules in human that are present in 41 usually nuclear proteins [16]. Bromodomains have been named after the *Drosophila* gene "brahma" for which the core bromodomain sequence motif was first identified [17]. The proteins that contain one or more bromodomains comprise histone acetyl transferases such as PCAF, GCN5L2, CREBBP and EP300, ATP-dependent chromatin remodelling factors (SMARCA2/4 and ATAD2A/B), the methyl transferase ASH1L, transcriptional modulators such as BRPF, TAF1/TAF1L, TRIMs, BETs and the nuclear body proteins (SP100, SP110 and SP140) [18] (Table 11.1, Fig. 11.1).

Table 11.1 Bromodomain containing proteins and their roles in cancer

Protein	Role in cancer	Reference
ASH1L	Mutations or copy number changes detected in lung cancer	[19]
ATAD2A/B	Overexpressed in prostate, breast and lung cancer and high expression levels correlate with poor prognosis in some cancers	[20–25]
	Coactivator of E2F transcription factors	
	Controls the expression of B-MYB, the histone methyltransferase EZH2 as well as other growth promoting proteins	
	Involved in estrogen-induced cell proliferation and cell cycle progression of breast cancer cells	
BAZ1A/B	Aberrant expression in colon cancer	[26]
BAZ2A/B	Chromosomal rearrangements in paediatric pre-B acute lymphoblastic leukaemia	[27]
BRD1	Chromosomal rearrangements detected in leukaemia with PAX5 transcription factors	[28]
BRD2	Interacts with chromatin binding domain in Kaposi's Sarcoma-associated herpesvirus (KSHV) latency-associated nuclear antigen 1 (LANA-1). BRD2 expression is downregulated in nasopharyngeal carcinoma cells	[29, 30]
BRD3	A chromosomal aberration involving BRD3 and NUT gives rise to a malignant subtype of midline carcinoma	[31]
BRD4	Translocation t(15;9)(q14;q34) with NUT gives rise to a BRD4-NUT fusion protein and development of carcinomas	[32–36]
	BRD4 controls expression of growth promoting genes such as myc, aurora kinase and Bcl2	
	Required for expression of target genes of the super elongation complex in MLL	
	Drives expression of FOSL1 in lung carcinoma	
	BRD4 is required for the replication of tumour viruses	
BRDT	Over-expression detected in non-small cell lung cancer and squamous cell carcinomas of the head and neck as well as of the oesophagus	[37]
BRD7	Tumour suppressor required for efficient transcription of a subset of p53 target genes	[38]
	Deletion or low expression levels detected in lung cancer	
BRD8A/B	BRD8 regulates cellular survival and sensitivity to spindle poisons in colon cancer	[39]
BRD9	DNA copy number changes in lung cancer	[40]
BRPF1	Regulates HOX gene expression	[41, 42]
	Component of the monocytic leukemia zinc finger protein MOZ/MORF complex	
BRPF3	Component of the monocytic leukemia zinc finger protein MOZ/MORF complex	[42]
BRWD3	Chromosomal aberration leading to the disruption of the BRWD3 locus has been detected in B-cell chronic lymphocytic leukemia (B-CLL)	[43]
CECR2	DNA damage response protein required for DSB repair	[44]

(continued)

Table 11.1 (continued)

Protein	Role in cancer	Reference
CREBBP	Chromosomal aberrations and mutations detected in acute leukaemia	[45, 46]
	Inhibitor of RUNX1-mediated transcription	
	Required for self-renewal of leukaemic stem cells	
	Cofactor of androgen receptor in prostate cancer	
EP300	Mutation identified in epithelial cancers	[47, 48]
	Chromosomal translocation detected in leukaemia	
FALZ	Overexpression is highly predictive of brain metastasis in early and advanced lung cancer	[49]
GCN5L2	None identified as yet	
MLL	Mutation chromosomal translocations give rise to acute lymphoid leukemias and acute myeloid leukemias	[50]
PB1	Tumour suppressor and regulator of p21 expression. Truncations and mutants in PB1 are a cause of renal cell carcinoma (RCC) and other cancer types	[51, 52]
PCAF	Inhibitor of the mitogenic activity of the adenoviral oncoprotein E1A	[53–55]
	Acetylates β-catenin enhancing its stability	
	Overexpressed in some paediatric tumours	
PHIP	Overexpression in an adverse predictor of survival in melanoma patients	[56, 57]
	Regulates PI3K/AKT signalling	
PRKCBP1	T-cell lymphoma antigene	[58]
SMARCA2A/B	Loss of function mutations and down-regulation of this tumour suppressor have been detected in multiple tumour types	[59–61]
SMARCA4	Germ line loss of function gives rise rhabdoid tumor predisposition syndrome type 2 (RTPS2)	[62]
	Mutation detected in many diverse cancers	
SP100/110/140	SP bromodomain proteins are part of promyelocytic leukemia (PML) nuclear bodies	[63–66]
	SP100 has tumour suppressor function that is lost in some cancer types	
	SP100 inhibits ETS1 DNA binding and regulates ETS1 transcriptional activity	
	SP140 has been identified as a susceptibility gene for CML	
TAF1/TAF1L	None identified as yet	
TRIM24/28/33/66	Overexpression of TRIM24 correlates with tumour progression in lung cancer and survival of breast cancer patients but promotes development of hepatocellular carcinoma by formation of a complex with TRIM33 and TRIM28	[67–71]
	TRIM33 is overexpressed in colon cancer	
WDR9	None identified as yet	
ZMYND11	Copy number alteration in hematologic cancers	[72, 73]
	Linked to BRCA2 pathway	

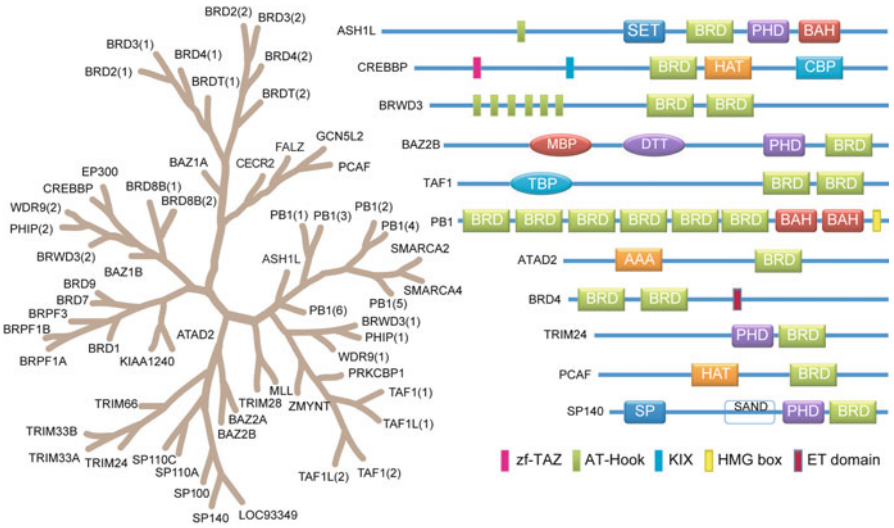

Fig. 11.1 Phylogenetic tree of the bromodomain family and domain organization of representative bromodomain containing proteins

11.2 Molecular Architecture of Bromodomains

Despite the low level of sequence conservation, particularly in the terminal helices, all bromodomains share a conserved fold that comprises a left-handed bundle of four alpha helices (αZ, αA, αB, and αC). The four bromodomain core helices are linked by highly diverse loop regions (ZA and BC loops) that flank the acetyl-lysine binding site and determine substrate specificity (Fig. 11.2). Acetyl-lysine containing sequence motifs bind to a large central cavity created by the four canonical bromodomain helices. The bromodomain acetyl-lysine binding site contains largely hydrophobic and aromatic residues in addition to a conserved asparagine that anchors the carbonyl group of acetyl-lysine by a hydrogen bond. A number of alternative residues exist that can potentially act as hydrogen bond donors, but no complexes with acetyl-lysine containing peptides that would confirm the role of these alternative residues have been reported so far.

11.3 Bromodomain Recognition Sequences

The multi-domain architecture suggests that the reading process of epigenetic marks is a modular process which may involve interactions with diverse modifications that are required to precisely recruit a certain regulator to a specific site on chromatin. However, epigenetic marks that are simultaneously recognized by these

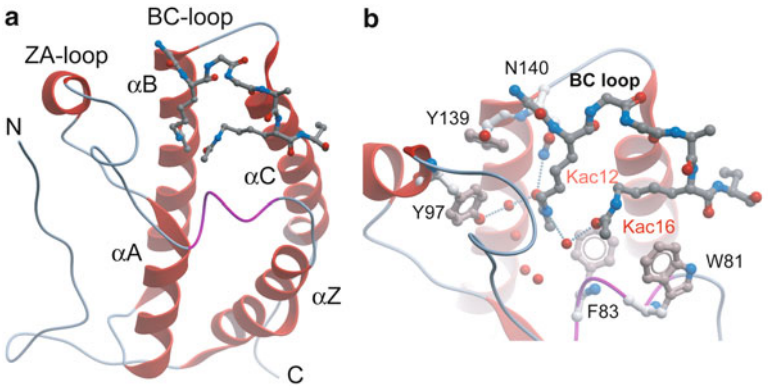

Fig. 11.2 Molecular architecture of bromodomains. (**a**) Shown is a peptide complex of the N-terminal bromodomain of human BRD4 with a diacetylated histone peptide. The main secondary structure elements are labelled and the N- and C- termini are indicated by N and C, respectively. (**b**) Details of the di-acetyl peptide interaction with the peptide binding site of BRD4. Peptide carbon atoms are highlighted in *grey* and acetyl-lysine residues on the H4 histone peptide are labelled in *red*. Water molecules are shown as *red spheres* and the main interacting residues are labelled in *black*

multi-domain proteins may be located in different histones, nuclear proteins or even on different nucleosomes. The complexity of the reading process poses a formidable challenge for our understanding of the epigenetic code.

Progress in peptide array technology has enabled systematic studies that have unravelled sequence specificity of individual reader domains. A recent comprehensive study revealed preferred histone interaction sequences for many members of the bromodomain family [16]. However, these data showed also that many bromodomains do not target histones and are probably recruited to other nuclear or non-nuclear protein complexes. For bromodomains that did interact with histones, another two interesting hallmarks of bromodomain peptide recognition were revealed: Firstly, many bromodomains seem to recognize poly-acetylated rather than singly acetylated lysine sites; and secondly post-translational modifications flanking the recognized acetylation site have a dominant effect on the bromodomain peptide recognition process.

Binding of a bromodomain to two acetylation sites was first reported for the bromodomain protein of murine BRDT [74]. Subsequent co-crystal structures and peptide array data showed that this is a shared feature of all N-terminal BET bromodomains [16] and significantly stronger interactions of poly-acetylated peptides with many bromodomains has suggested a widely distributed recognition mode for diacetyl-lysine containing sequences. A number of co-crystal structures of diacetyl-lysine containing peptides with BRD4 revealed a conserved interaction of both acetyl-lysines with the bromodomain peptide binding pocket. In all co-crystal structures the conserved bromodomain asparagine (N140 in BRD4(1)) forms a hydrogen bond with the carbonyl of the N-terminal peptide acetyl-lysine as it has

been described for mono acetylated peptide complexes. In contrast the second acetyl-lysine carbonyl forms no direct polar contacts with the bromodomain but interacts via a water-mediated hydrogen bond with a conserved tyrosine residue (Y97 in BRD4(1)). Together both acetyl-lysine side chains show remarkable shape complementarity with the peptide binding pocket. No structural information of peptide complexes with additional post translational modifications have been reported so far. It is likely however that differences in surface potential (e.g. additional negative charges contributed by phosphorylated residues) significantly influence the recognition process of bromodomains for their recognition sequences. This feature suggests that bromodomains interpret post translational modifications generated by diverse pathways and function therefore as nuclear integrators of cellular signalling.

11.4 Development of Bromodomain Inhibitors

The acetyl-lysine binding pocket in bromodomains represents an attractive site for the development of inhibitors. In contrast to unmodified lysine side chains the acetylated residue is not charged. Thus, binding pockets that specifically recognize this PTM contain mainly neutral, hydrophobic and aromatic residues allowing the development of cell permeable acetyl-lysine competitive drug-like molecules. The size of the binding pocket, its high level of enclosure and its residue composition result in good predicted druggability values for most bromodomains [75]. However, inhibitor development efforts have focussed on few bromodomains so far.

The first bromodomain inhibitor, NP1, was described in 2005 by the Zhou laboratory as an inhibitor of the PCAF HIV-1 TAT interaction (PCAF IC_{50} 1.6 μM) [76]. Subsequently a number of weakly binding fragments (MS2126 and MS7972) binding to the CREBBP bromodomains were discovered by the same laboratory using NMR screening methods and a library of putative acetyl-lysine mimetic compounds [77]. The most potent inhibitor of this series (MS7972) effectively repressed CREBBP recruitment to p53 suppressing p53 transcriptional activity demonstrating that bromodomains can be efficiently targeted in the cellular context. Interestingly NP1 and MS7972 do not seem to bind in an acetyl-lysine mimetic binding mode as they form no hydrogen bond with the conserved asparagine that typically ligates the acetyl-lysine carbonyl in peptidic complexes of bromodomains and their target sequences. Further development of the MS series led to ischemin which showed an IC_{50} of 19 μM for CREBBP. Ischemin however mimics the acetylated lysine binding mode by forming a hydrogen bond to N1168 of CREBBP [78]. The tetrahydroquinoline Example 6 in WO2011/54848 can be seen as a larger analogue of MS2126 [79]. The acetyl-lysine mimetic binding mode has also been demonstrated in complexes of the fragment 3-methyl-3,4-dihydroquinazolin-2-(1*H*)-one with CREBBP and the BET inhibitor BIC [80].

Highly potent inhibitors were initially reported in the patent literature as potent BET inhibitors, a discovery that led to the development of the pan-BET thienodiazepine bromodomain inhibitor JQ1 [81] and benzodiazepines such as I-BET [82, 83].

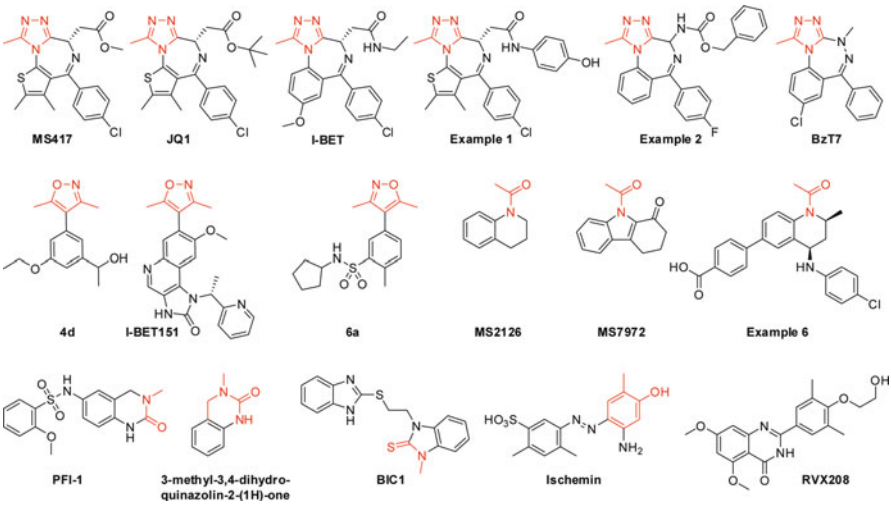

Fig. 11.3 Structures of known bromodomain inhibitors. In cases where experimental co-crystal structures have been determined we highlighted the acetyl-lysine mimetic moiety in *red*. Example 1 and 2 are from the patent WO2009/84693, WO/2011/054844, respectively and Example 6 (tetrahydroquinoline) has been described in the GSK patent application WO2011/054848. Compound 4d and 6a have been published in the paper by Hewings *et al*. All other inhibitors are described in the main text

Inhibitors of this class utilise the methyltriazolo-diazepine ring system as the acetyl-lysine mimetic. I-BET and JQ1 contain a stereo centre which makes synthesis or separation of the stereoisomers a challenging task. This problem was overcome by the development of benzotriazepines (for example Bzt7) where the asymmetric carbon atom is replaced by nitrogen [84]. Substitution of the methyltriazolo with the isosteric methylisoxazoles led to the development of a number of isoxazole based inhibitors with good ligand efficiency and potency [85, 86]. The most optimized inhibitor of this series, I-BET, showed good antiproliferative potency in cells as well as in mouse models of leukaemia [32]. A compilation of currently known bromodomain inhibitors is shown in Fig. 11.3.

11.5 Chromosomal Aberrations Lead to Oncogenic BRD Fusion Proteins

A number of bromodomains have been identified in highly oncogenic fusion proteins. One of the best characterized cancers with chromosomal translocations is NUT midline carcinoma (NMC), a highly aggressive rare, poorly differentiated squamous cell carcinoma, which predominantly occurs in children and mainly arises in the midline of the body, commonly in the head, neck or mediastinum. More

recently BRD-NUT oncoproteins have also been detected in cancers with different tissue origin [33]. NMC is unusual because the single chromosomal translocation is often the only genetic aberration found in this cancer. In the majority of cases it is characterized by the rearrangement of the nuclear protein in testis (*NUT*) gene, located on chromosome 15q14 and the *BRD4* gene on chromosome 19p13.1 to create a *BRD4-NUT* fusion gene [87] whose product is driven by the *BRD4* promoter. The *BRD4-NUT* fusion gene contains the N-terminus of BRD4 or in rare cases BRD3 including both bromodomains and essentially the entire coding region for NUT [33, 88]. The NUT portion of the gene product is thought to be largely unstructured and contains an acidic binding domain for the HAT EP300. Little is known about the function of the NUT protein, though under normal conditions it is known to shuttle between the nucleus and the cytoplasm. When NUT is fused to BRD4 or BRD3 however it remains in the nucleus [31]. This implies that the BRD portion of the fusion protein tethers NUT to chromatin, affecting the function of one or both proteins and resulting in a knock-on effect on transcription. Genetic knock-down of BRD-NUT by siRNA in NMC cells results in dramatic terminal squamous differentiation and in G1 growth arrest consistent with the notion that BRD-NUT maintains an undifferentiated state of NMC cells [88, 89].

Another chromosomal aberration results in the disruption of the *EP300* and *CREBBP* locus in leukaemia [90]. The first of these translocations characterized was the t(8,16)(p11,p13) translocation which fuses the gene encoding the monocytic leukemia zinc-finger protein (MOZ) with the amino terminus of CREBBP, giving rise to the M4/M5 subtype of acute myeloid leukaemia (AML) [91, 92]. This translocation may result in activation of the HAT domain in the fusion protein, however the t(8,16) translocation is rare, accounting for only 0.4 % of patients with AML. A number of MOZ fusions with the CREBBP related HAT EP300 have been also described t(8,22)(p11,q13) [93, 94]. Fusion of the mixed lineage leukaemia gene products (MLL) with CREBBP and EP300 have been reported in relapsed MLL after treatment with topoisomerase inhibitors [95, 96]. *CREBBP* is more commonly involved in chromosomal rearrangements than *EP300*. Interestingly most of the *CREBBP* rearrangements target the same 13 kb genomic interval suggesting that this region contains an unstable genomic element accounting for the higher frequency of *CREBBP* rearrangements [97].

11.5.1 Aberrant Expression of Bromodomain Proteins in Cancer

A number of genes coding for proteins containing bromodomains are overexpressed in cancers and expression levels have been linked to tumour progression and in some cases patient survival. An interesting example is TRIM24/TIF1α which has been reported as a tumour suppressor in liver cancer but as a tumour promoting factor in breast and lung cancer suggesting highly context dependent roles of reader domains.

Overexpression of TRIM24 has been shown to correlate with poor survival of breast cancer patients [67]. The expression of TRIM24 has also been examined in non-small-cell lung (NSCLC) cancer tissues where high expression levels were found in tumour cells compared to normal lung epithelium [68]. Cells from patients with high levels of TRIM24 expression were largely undifferentiated and showed high proliferation activity. Interestingly, TRIM24 levels did increase in patients with advanced NSCLC. Knockdown of TRIM24 by siRNA inhibited cell cycle progression, slowing proliferation and inducing apoptosis. TRIM24 has also been linked to chronic myeloid leukemia (CML) where expression was found to be low in the chronic phase but increasing gradually through the accelerated phase to the blastic phase [98]. This pattern of expression suggests that it is intrinsic to the progression of the disease. In the same study it was also observed that TRIM24 was overexpressed in the CD34-positive compartment, suggesting that the signalling pathways governing CML progression and CML stem cell homeostasis may be related.

In contrast, TRIM24 has also been reported as a potent liver-specific tumour suppressor. TRIM24 knockout mice are predisposed to the development of both spontaneous and chemically induced hepatocellular carcinoma [69]. Studies in TRIM24$^{-/-}$ mice showed upregulated retinoic acid receptor signalling, an effect that is compensated by simultaneous mono allelic deletion of the *Rara* gene [70].

BRDT has been associated with a number of different cancers. This usually testis specific BET family member has been found to be overexpressed in digestive tract tumours [99] and in non-small cell lung cancer where BRDT was found to be expressed in nearly half of the biopsies examined [37].

Overexpression of ATAD2 has been reported in a large variety of tumours, particularly in gastrointestinal tumours, large B-cell lymphoma, hepatocellular carcinoma and breast and lung cancers [100–103]. ATAD2 overexpression correlates with poor prognosis for lung and breast cancer patients and high expression levels have also been linked to distant recurrence [104]. Significantly, ATAD2 is highly expressed in >70 % of breast tumours and its expression correlates with highly aggressive, triple negative tumours, tumour metastasis and poor prognosis for the patient [20].

ATAD2 has been shown to bind to histone H4K5ac through its bromodomain, and to the transcription factor c-MYC (v-myc myelocytomatosis viral oncogene homolog) stimulating transcription of c-MYC target genes [105]. It is also a mediator of E2F transcription factors and is required for recruitment of the host cell factor 1 (HCF-1)-MLL histone methyltransferase complex to chromatin. In agreement with its key function regulating growth promoting transcription factors, genetic knockout studies showed that Atad2 is essential for proliferation and survival of tumour cells and for promoting cell proliferation, survival and cell migration.

11.6 Tumour Suppressor Roles of Bromodomain Proteins

The gene encoding for the central component of SWI/SNF (Switch/Sucrose non-fermenting) chromatin remodelling complexes SMARCA4 (/BRG1, Brahma related gene) was identified as a tumour suppressor gene [106, 107]. *SMARCA4* expression

is silenced in several human tumour cell lines and tumour tissues and inactivating mutations have been detected in rhabdoid tumours. However, the frequency of SMARCA4 loss varies significantly across different tumour types and it is particularly frequent in lung cancer [106, 108]. Interestingly, *SMARCA4* expression never occurs concurrently with *cMYC* amplification, suggesting that these factors may play a common role in lung cancer. SMARCA4 was subsequently shown to inhibit c-Myc-mediated transcription in HeLa cells [109]. Loss of SMARCA4 has been observed to correlate with lung cancer tumour aggressiveness [110] and SMARCA4 inactivation is often accompanied by loss of the related gene product SMARCA2 (/BRM, Brahma gene) [111]. The combined loss of the ATPase activity of both proteins may lead to the observed aggressive phenotype of these tumours. Furthermore, cells that express a mutant SMARCA4 gene lacking ATPase activity increase in both overall volume and nuclear size and also in area of attachment [112]. However SMARCA4 and SMARCA2 are not functionally redundant, despite having a high degree of homology and several overlapping functions [108]. Recently SMARCA4 has been implicated as a tumour suppressor in melanoma, binding to $p16^{INK4a}$, an important melanoma susceptibility gene [113]. Other components of the SWI/SNF complexes are also frequently truncated, mutated or silenced in tumours. These components include the polybromo protein PB1, a component of the PBAF (Polybromo and Brg1-associated factor) subclass of SWI/SNF complexes which has also been found to be mutated in a high percentage of renal cell carcinomas. In support of its role as a tumour suppressor, genetic knockdown of more than 60 % of PB1 resulted in significant increase in proliferation in renal cell carcinoma cell lines.

11.6.1 Bromodomain Proteins as Driver of Genetic Programs of Tumourigenesis

A number of bromodomain-containing proteins are key regulators that drive transcriptional programs that lead to cell proliferation. An interesting example of such a central regulatory function is the BET family member BRD4, which is required for efficient transcriptional elongation of diverse growth-promoting and anti-apoptotic genes. BRD4 and other BET family members recruit the positive transcription elongation factor complex (P-TEFb, cdk9/cyclinT) to transcriptional start sites during the M/G1 transition allowing CDK9 to phosphorylate the C-terminal domain of RNA polymerase. This leads to a unique marking of genes that remain to be expressed at the end of mitosis, functioning as a component of transcriptional memory during cell division. Intriguingly, genes that are controlled during cell division in a P-TEFb/BRD4 dependent manner include the mitotic kinase Aurora B, NF-κB and c-MYC as well as anti-apoptotic genes such as BCL2. Recently Zuber and colleagues showed that BRD4 is required for AML cell survival by upregulating a number of growth stimulating genes such as c-MYC providing a compelling strategy for targeting BRD4 in leukaemia. Chemical inhibition of the BRD4 bromodomain by the BET inhibitor JQ1 also resulted in significant antiproliferative effects, down-regulation of c-MYC transcription, cell-cycle arrest and cellular

senescence in multiple myeloma. Similar to other bromodomain proteins the effect of BET inhibition on transcription is highly context dependent. In lung cancer for instance c-MYC levels are not affected. Instead BET inhibition has been linked to another set of affected genes that include the oncogenic transcription factor FOS-like antigen 1 (FOSL1). In breast cancer however, *BRD4* has been identified as a susceptibility gene for disease progression. In this cancer high expression levels have been associated with poor patient survival presumably due to transcriptional up-regulation of genes that promote cell migration and as a consequence metastasis. Similar to hormone receptors bromodomain-containing transcriptional regulators need to be comprehensively studied in different tissues to evaluate their role in normal physiology and disease. The excellent druggability of these domains however, together with their central role in tumourigenesis suggests that many bromodomain containing proteins will develop into attractive drug targets in the future.

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Chapter 12
Domain Specific Targeting of Cancer

Pratik Chandrani and Amit Dutt

Abstract Cancer is driven by sequential accumulation of genetic alterations leading to the gain- and loss-of-function of critical cellular proteins. Genes of several families (kinases, phosphatases, RAS, GPCRs, etc.) with similar functions, as predicted by similar coding sequences and protein domains, are known to be significantly altered in cancer. Targeted therapy directed against the activity of specific gene products that confer "oncogene addiction" to tumor cells minimizes general toxicity associated with the nonspecific chemotherapy treatments. The success though is restricted to a smaller subset of patients due to development of resistance. One solution to the enduring challenge of drug resistance is rational combinatorial targeted therapy targeting multiple domains across gene families.

Keywords Targeted therapy • Kinase • Phosphatase • Small GTPase • Resistance to targeted therapy

12.1 Introduction

Cancer, as we understand today, is a disease of the genome driven by specific alterations that may be inherited in the germ-line or acquired somatically. These genome alterations principally include copy number alterations, structural re-arrangements, nucleotide substitution mutations, and infection by microbial genomes leading to the gain- and loss-of-function of critical cellular proteins. Activated oncogenes conferring oncogenic addiction to cancer cells for maintenance of its malignant

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phenotype, are the Achilles' heel for the cells. The cause of addiction could thus be an effective therapeutic target to kill the addicted cancer cells, a prototypical example being the use of Gleevec [1], a small-molecule inhibitor of the Bcr-Abl fusion protein in chronic myelogenous leukemia. Several additional examples of such alterations that potentially define therapeutic paradigm include: *ERBB2* amplification in breast cancer; *EML-ALK* fusion in lung cancer and *ALK* mutation in Neuroblastoma; point mutation, insertion or deletion of *EGFR* in lung cancer; *KIT* mutation in gastrointestinal tumors; *BRAF* mutation in melanoma; *JAK2* mutations in myeloproliferative disorders, *FGFR2* mutation in endometrial cancer, *IDH1* mutation in glioblastoma, and *PIK3CA* in several cancers. Effective development of targeted therapeutics that can interfere with the function of oncogenic molecular targets though remains unmet due to lack of our understanding of the molecular alterations that drive the tumorigenesis in different cancers. While each cancer subtype harbors its own unique pattern of genomic lesions, comparison of genomic lesions across cancer subtypes generate a global view of the disease associated genomic alterations. Besides informing therapeutic regimen in clinics, the elucidation of the function of cancer related genes has significantly enhanced our understanding of the developmental biology of normal cells as the fundamental biochemical pathways deregulated in cancer cells has uncovered various normal homeostatic roles such pathways play in non-transformed cells and tissues. A comprehensive catalog of all such genetic lesions, together with a description of the cancer types in which they occur and the combinations in which they co-occur, thus is necessary for the effective development of targeted cancer therapy. Interestingly, most frequently mutated genomic alterations which governs majority of cancer types lies into some of the most common pathways, for example oncogenic activations of diverse kinase proteins (*EGFR*, PDGFR, *ERBB2*, FGFR, *ABL1*, etc.) and downstream components all together leads to activation of RAS/RAF/MAPK/ERK pathway affecting deregulation of cell growth. Most of the inactivating alterations in tumor suppressor genes affect the CDKN2A/CDK/Rb/E2f and WNT/P53/CDK/Cyclin/ mediated cell cycle and apoptosis pathways. Thus, several pathways and pathway components have more profound effect on cellular deregulation. In this chapter, we will discuss these most frequently altered proteins, their functional domains and targeted therapy in cancer.

12.2 Major Protein Families Mutated in Human Cancer

The cellular necessity of a drug target to cancer versus normal cells dictates the width of the therapeutic window, as inhibiting a crucial cancer-specific target may as well jeopardize the viability of normal cells. The ideal drug target would be essential to tumors, yet non-essential to normal cells. Among the most widely recognized genetic alterations are those that lead to the activation of regulatory cascades that are governed by the biochemical activity of cellular kinases. These genetic alterations take the form of the direct mutation of kinase genes through

translocation, missense mutation, nonsense mutation, gene amplification or gene deletion. Indeed, the very first oncogene, v-src, and the first genetic lesion identified in human cancer, t(9;22), targeted tyrosine kinases. Then, the *MYC* proto-oncogene was found to be activated by translocation as well as amplification, and amplification thus became recognized as an additional cardinal mechanism of cancer gene deregulation. One of the first pivotal discoveries of activating mutations was within *BRAF*, which encodes a serine/threonine kinase oncogene that transmits proliferative and survival signals downstream of *RAS* in the mitogen-activated protein (MAP) kinase cascade followed by receptor tyrosine kinases (*EGFR*, *ERBB2*, FGFR1, *FGFR2*, FGFR3, PDGFRA, PDGFRB, *ALK*, c-MET, IGF1R, c-KIT, FLT3, and RET), non-receptor tyrosine kinases (ABL, *JAK2*, and SRC), serine-threonine-lipid kinases (*BRAF*, Aura A and B kinases, mTOR, and PIK3), and DNA damage and repair genes (BRCA1 and BRCA2). Timeline for discovery of mutations in few of these therapeutically relevant genes is shown in Fig. 12.1.

Besides, a larger set in the human genome encode proteins termed the druggable genome. The human druggable genome consists of 5,520 different genes distributed through various gene families including serine/threonine and tyrosine kinases (22 %), G-protein-coupled receptors (15 %), cation channels (5 %) serine proteinases (4 %), protein phosphatases (4 %) and other (50 %). Other includes, but is not limited to, zinc peptidases, cytochrome P450s, and nuclear hormone receptors. While the ongoing discovery of the genetic basis of cancer has allowed us to leap forward in our understanding of cancer biology, these developments suggest strongly that a genetic footing for cancer coupled with a mechanistic and biological understanding of the downstream consequences can beget novel effective therapeutics. Following is an overview of few selected major protein families involved in human cancer:

12.2.1 RAS Family

RAS family of oncogenes is the earliest oncogene family identified and validated for oncogenic addiction. Oncogenes *HRAS*, *KRAS* and *NRAS* are the founding member of *RAS* family. *RAS* is a membrane-associated guanine nucleotide-binding protein that is normally activated in response to the binding of extracellular signals such as growth factors to Receptor Tyrosine Kinases. *RAS* signaling controls many cellular functions including cell proliferation, apoptosis, migration and differentiation. *RAS* family includes 35 members of proteins, a subset of more than 150 members in GTPase family. All the *RAS* onco-proteins are reported to be having activating mutations (gain-of-function) in different types of cancers, mainly colorectal, lung, pancreatic and cervical cancers. The three *RAS* onco-proteins all together constitute oncogenic addiction in ~30 % of the cancers [2, 3]. Mutational screening of *RAS* genes in large number of tumor samples shows a repertoire of cancer associated mutations. Although there are several different mutations reported in all three oncogenic *RAS* proteins, there is a selective bias observed for some mutational hotspot sites (Fig. 12.2). As reported in cosmic database (as on date: December 2012),

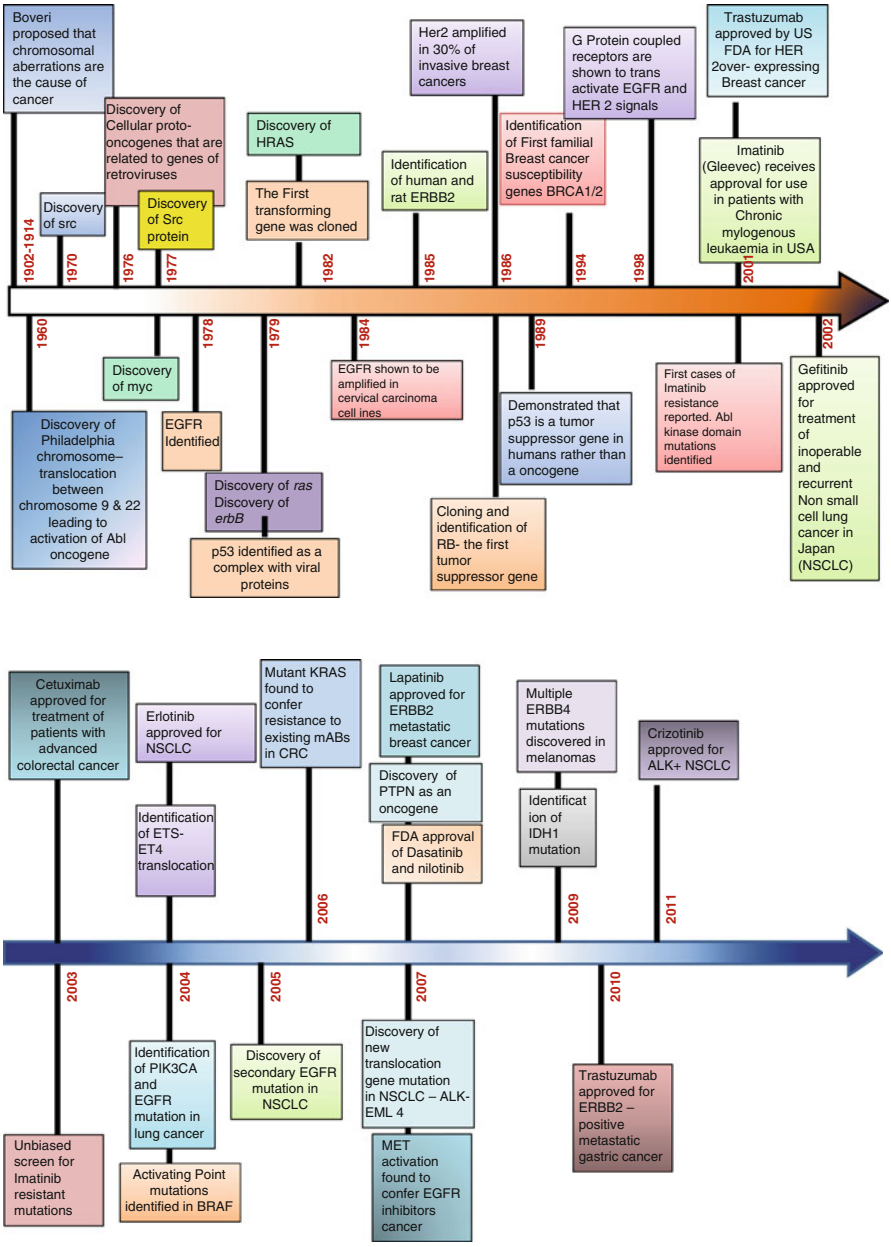

Fig. 12.1 Schematic representation of timeline for discovery of domain specific mutations in human cancer

RAS proteins have three hot-spot positions, G12, G13 and Q61 which are hyper mutated. Selective pressure for these mutation sites is understandable since these sites are directly involved in activation of *RAS* protein. Complex interactions between G12 and Q61 with GTPase activating protein (GAP) induce

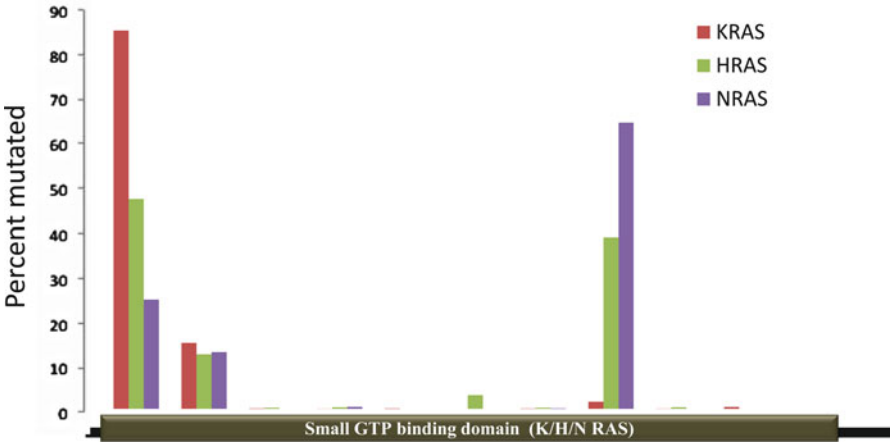

Fig. 12.2 Domain specific mutation frequency among Ras family proteins in human cancer

conformational changes, making *RAS* into active conformation. Impaired ability of *RAS* mutants to hydrolyze GTP is responsible for the oncogenic nature of mutations at residues G12, G13, and Q61 in the active site. Having a conserved mutation patterns can be of great aid in therapeutic applications. Unlike tyrosine kinase targeted therapies, *RAS* targeted therapy has seen limited success. The most thoroughly investigated anti-RAS compounds are the farnesyl transferase inhibitors (FTIs) [4], which inhibit the post-translational modifications of *RAS* by a farnesyl isoprenoid group. Another distinct approach has been the compounds blocking *RAS* interaction with membrane, Trans-farnesyl thiosalicylic acid (FTS) [4], which blocks interaction of *RAS* from *RAS* anchorage domains in the cell membrane. The main reasons for limited success in *RAS* targeted therapy is given by nature of target, anti-RAS drugs need to restore GTPase functionality of mutated *RAS*. Other reasons for limited anti-RAS therapies are many other downstream pathways which might not be totally dependent on *RAS*, for example PI3-K pathway can be activated by both *RAS* dependent and independent mechanisms [2, 3]. Alternatively, scientists are taking alternative approach for targeting *RAS* by blocking downstream pathway and by taking novel approach of fragment based drug designing. This work is still at very early stage and confirmed reports are awaited.

12.2.2 Kinases

Protein kinase (PK) is an enzyme, which modifies other proteins by adding phosphate group to them. Phosphorylation of target protein results in functional change in target protein by changing enzyme activity, cellular localization or protein-protein interactions. There are about 518 kinases and 106 kinase pseudo-genes in human genome (~2 % of human proteome) [5] which plays vital roles in regulation

of proteins involved in cell growth and proliferation, cell cycle regulation, metabolism, apoptosis and various other signal transduction pathways. All human protein kinases constitute a complicated network of regulatory proteins with external and internal regulatory connections. Alteration in any node of this network is vulnerable to disruption of regulatory network due to the complexity and sophistication of the system. Alteration in kinases has been shown to be affecting several physiological processes resulting into malignancies including cancers.

Systematically kinases are divided into ten groups: (1) protein kinase A, G, and C family altogether called as AGC kinases, (2) cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAP kinases), glycogen synthase kinases (GSK) and CDK-like kinases altogether make CMGC group of kinases, (3) Calmodulin/Calcium regulated kinases (CAMK), (4) Casein Kinase 1 (CK1) group, (5) STE group of kinases, (6) tyrosine kinases (TK), (7) Tyrosine kinase like (TKL) generally phosphorylating serine/threonine residue, (8) Receptor Guanylate Cyclases (RGC) group of kinases, (9) protein kinase like (PKL) proteins having fold similar to kinases and (10) atypical protein kinases (aPK) consist of proteins which do not share clear sequence similarity with other kinases but are functioning kinase like. We can also broadly classify all PKs as receptor protein kinases (RKs) which contains trans-membrane domain and cytoplasmic protein kinases (CKs) which are found in cytosol, nucleus and inner surface of plasma membrane.

Although PKs are much diversified and are divided into several groups, they all share a significant sequential and structural similarity that is understandable as they all transfer phosphate group of ATP to serine, threonine or tyrosine residue of target protein. All kinases share a common mechanism of catalysis where phospho-transfer is carried out by shared set of amino acids and ATP & active site divalent cation are bound in similar fashion. This similarity and divergence suggests that PKs share a common domain for general function of catalysis and harbor other domains for substrate specificity to the target protein. The domain architecture of representative receptor kinases and non-receptor kinases is much similar to each other.

Alterations in protein kinase' domains have been shown to be playing role in tumorigenesis by creating a genomic change which affects functionally important amino acids. The most commonly observed changes in PKs are activating mutations which lead to deregulation of cell growth and proliferation. Constitutive activation of these proteins gives advantage of uncontrolled growth to cancer cells, and so inhibition of these mutant proteins can be a therapeutic option. Small molecules or antibodies can be designed for mutant proteins and thus can be selective for mutant only. The first example of targeted therapeutic for a mutant protein is Imatinib (Gleevec) against *ABL1* gene in patients with refractory chronic myelogenous leukemia (CML) [1, 6]. Later, imatinib was used to treat advanced-stage gastrointestinal stromal tumors (GIST) carrying activating mutations in two tyrosine kinase receptors, *KIT* and PDGFR [7]. The relationship of imatinib response and *KIT* mutations developed a new paradigm which encouraged development of more targeted therapeutics. Another best model of oncogenic addiction and targeted cancer therapy is of tyrosine kinase inhibitors (TKIs) against epidermal growth factor receptor (*EGFR*) in non-small-cell lung cancer (NSCLC). *EGFR* is founding

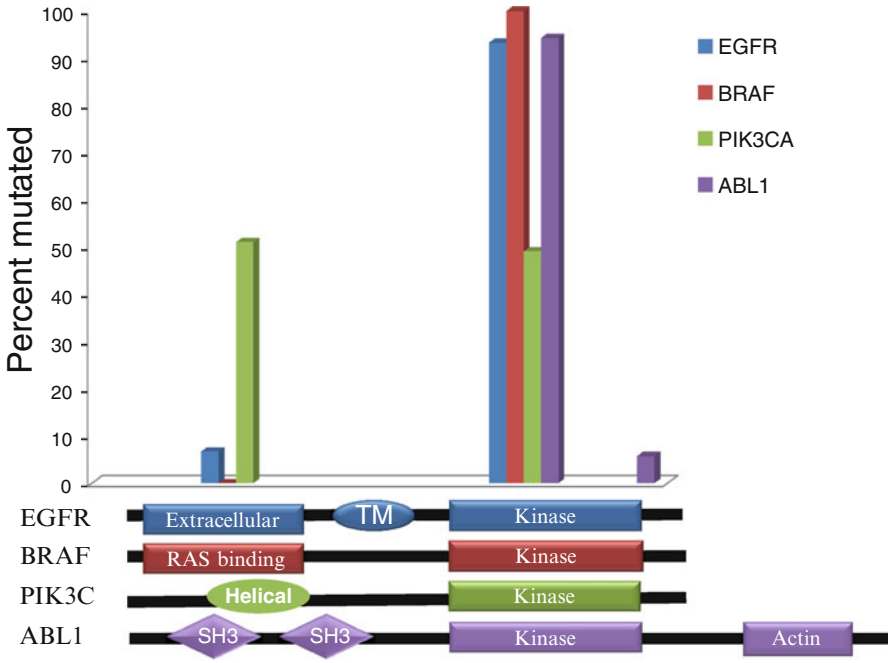

Fig. 12.3 Domain specific mutation frequency among Kinases in human cancer

member of ErbB family, identified as an oncogene in 2004. Along with other three members of ErbB, *EGFR* has been of intense research for targeted therapeutics. Alterations in ErbB family of genes are reported in many cancers including lung, glioblastoma, thyroid, head and neck carcinoma etc. [8, 9]. Most of oncogenic *EGFR* mutations in NSCLC are located in kinase domains (exon 18-21) which constitutively signals the PI3K/Akt/mTor pathway to promotes cell growth, survival, and migration as well as resistance to apoptosis in response to *EGFR* mediated activation [10]. In the absence of therapy, patients with lung cancer who harbor an *EGFR* mutant tumor have reported better prognosis compared with patients with wild type *EGFR* [11]. Even though prevalence of *EGFR* mutations vary greatly between different ethnicity ranging from ~10 % in Europe and USA to ~35 % in Asia, *EGFR* specific inhibitors has shown successful response in clinical studies [12]. Erlotinib and gefitinib are the two targeted inhibitors designed for *EGFR* mutants which binds to ATP binding site of kinase domain and blocks downstream signaling pathway.

Genomic analysis of large number of patients across the globe shows highest density of mutation at selective sites of *EGFR*. As shown in Fig. 12.3, mutation frequency per amino acid position of *EGFR* (from COSMIC databases) shows clear preference for few selective sites. Maximum frequency of mutations are observed for L858, G719, TL861, T790 and small deletions of 4–6 base pairs in exon-19 (Fig. 12.3). This selective mutation pattern is also observed for other kinases, for

Table 12.1 US FDA approved small molecule inhibitors and antibodies against various protein kinases for treatment of cancer

Drug	Targets	US FDA approval for cancer subtype
Bosutinib	BCR-ABL	CML
Crizotinib	EML4-ALK	Lung cancer
Dasatinib	BCR–ABL	CML
Erlotinib	*EGFR*	Lung and pancreatic cancers
Everolimus	mTOR	Kidney cancer
Gefitinib	*EGFR*	Lung cancer
Imatinib	BCR-ABL, PDGFR and *KIT*	CML and GIST
Lapatinib	*EGFR* and *ERBB2*	Breast cancer
Nilotinib	BCR–ABL	CML
Pazopanib	VEGFR2, PDGFR and *KIT*	Kidney cancer
Sorafenib	VEGFR2 and PDGFR	Kidney and liver cancers
Sunitinib	VEGFR2, PDGFR and *KIT*	Kidney cancer and GIST
Temsirolimus	mTOR	Renal cancer
Vandetanib	*EGFR*, VEGFR, RET	Medullary thyroid cancer
Vemurafenib	*BRAF*	Melanoma
Antibodies	**Targets**	**US FDA approval for cancer subtype**
Bevacizumab	VEGF	Colorectal, lung and breast cancers
Cetuximab	*EGFR*	Colorectal, and head and neck cancers
Panitumumab	*EGFR*	Colorectal cancer
Pertuzumab	HER-2	Breast cancer
Trastuzumab	*ERBB2*	Breast cancer

BCR breakpoint cluster region, *ABL* abelson murine leukemia viral oncogene homolog, *CML* chronic myeloid leukaemia, *EGFR* epidermal growth factor receptor, *FDA* Food and Drug Administration, *GIST* gastrointestinal stromal tumour, *PDGFR* platelet-derived growth factor receptor, *VEGFR2* vascular endothelial growth factor receptor 2, *ERBB2* erythroblastic leukemia viral oncogene homolog, *RET* rearranged during transfection

instance *BRAF*, *PIK3CA* and *ABL1* all shows very high preference for mutations in kinase domain (Fig. 12.3). Selectivity of mutation sites in different members of same protein family shows a selective pressure on cancer cells which is satisfied only by mutations at some particular site. These high prevalent mutations of kinases make them feasible target for small molecule inhibitors. Several small molecule inhibitors and antibodies for kinase family are already approved by Food and Drug Administration (FDA) or are under clinical studies (Table 12.1).

12.2.3 Phosphatases

A phosphatase is an enzyme which functions exactly opposite to kinases, by removing phosphate group from target protein. The state of phosphorylation of a protein

at a time is the net result of the opposing activities of the relevant kinases and phosphatases. Phosphatases and kinases all together make a cycle of target protein activation-deactivation resulting into controlled cellular signaling pathways. Generally a balance of protein phosphorylation-dephosporylation cycle is skewed towards dephosphorylated state, making a constant check on active form (phosphorylated) of target proteins. Hence, phosphatases often play very specific, highly regulated, and very active roles in many cellular processes.

There are approximately 200 protein phosphatases predicted in human genome. Phosphatases can be broadly divided into six classes on the basis of sequence of catalytic domains: (1) protein tyrosine phosphatases (PTP), (2) protein serine/threonine phosphatases (PPP), (3) Protein phosphatase 2C-like phosphatases (PPM), (4) Haloacid dehalogenase-like phosphatases (HAD), (5) phosphatidic acid phosphatase, inositol monophosphatase and inositol polyphosphate-related phosphatase collectively called LP phosphatases and (6) NUDIX hydrolase (NUDT) phosphatases. All the phosphatases can also be grouped as receptor protein phosphatases and cellular protein phosphatases.

Role of phosphatases in cancer is not extensively studied like kinases but it's observed that most of the phosphatases act as tumor suppressors. With some exceptions to the rule, the main function of phosphatases is to control the generally active (phosphorylated) form of target proteins by dephosphorylating (generally inactive form) them. Thus, in normal conditions, phosphatases suppress the oncogenic behavior of certain proteins. Several alterations are observed in phosphatases, mostly in-activating alterations which lead to uncontrolled activation of target protein. Therapeutic applications of inactivating alterations are very limited with only options of replacing defective protein form by gene-therapy or other similar techniques which are difficult to effectively utilize in regular practice.

Interestingly, protein phosphatases are also reported to potentiate, rather than antagonize the oncogenic action of protein kinases. Overexpression of PTP has been observed to activate Src oncogene by dephosphorylating inhibitory T527 residues of Src [13, 14]. Direct role of PTP as oncogene was first reported for *PTPN11* which encodes Shp2 protein [15]. A number of activating (gain of function) mutations in SHP2 domain has been identified as the cause of the Noonan syndrome, several forms of leukemia and solid tumors [16, 17]. Another phosphatase known for oncogenic activities is cell cycle regulator protein Cdc25. *Cdc25* has been implicated in the oncogenesis of breast cancer, prostate cancer, and non- Hodgkin's lymphoma, in which elevated expression of *Cdc25* is thought to promote the loss of cell cycle check-point control resulting into uncontrolled cell proliferation, and genetic instability [18, 19].

Just like kinases, analysis of mutation distribution in different phosphatases shows selective hot-spot sites for mutations. As given in Fig. 12.4, mutation distribution in oncogene *PTPN11* (from COSMIC) shows mutational hot-spot in SH2 domains. Similar types of hot-spots are also observed for inactivating mutations in PTEN, a known tumor suppressor. Selective mutational hot-spot makes phosphatases an important target for cancer therapies.

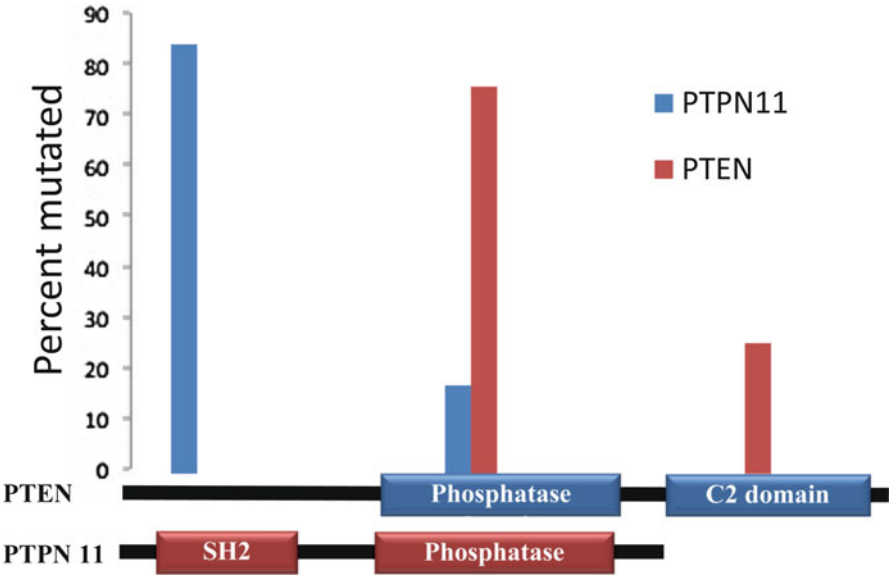

Fig. 12.4 Domain specific mutation frequency among Phosphatases in human cancer

12.3 Acquired Resistance to Domain Specific Inhibitors

Many of the targeted small molecule inhibitors show successful response in tumors driven by single oncogenic mutation. However, many tumors are driven by interaction of multiple oncogenic mutations, and therefore benefit of mono-targeted therapies is limited. Another limitation of targeted therapy is acquired resistance in tumor. Higher genomic instability in cancer cells gives rise to acquired resistance for targeted therapeutic drug. Two known mechanisms of acquired resistance are (1) secondary mutations in target protein and (2) mutation in downstream pathway of target protein. Both types of acquired resistances are reported for *EGFR* targeted therapies. Lung adenocarcinoma patients having L858R mutations or small deletions in exon-19 (near codon 745-750) were reported to be having ~90 % response to targeted inhibitors erlotinib and gefitinib. Soon after first application of these targeted drugs, some patients showed reversion of tumor. Genomic analysis identified secondary mutation T790M in *EGFR* [8, 20]. This mutation changes threonine to methionine in ATP binding site, which leads to lesser binding affinity for drugs while ATP binding not affected. Later, another mechanism of resistance was observed for *EGFR* TKIs as amplification in *MET* gene. Amplification of *MET* leads to sustained phosphorylation of ERBB3, which in turn activates PI3K-Akt signals downstream. In another scenario, activating mutation in PI3K also activates PI3K-Akt pathway. Thus, even though TKIs inhibits *EGFR*, alteration in another

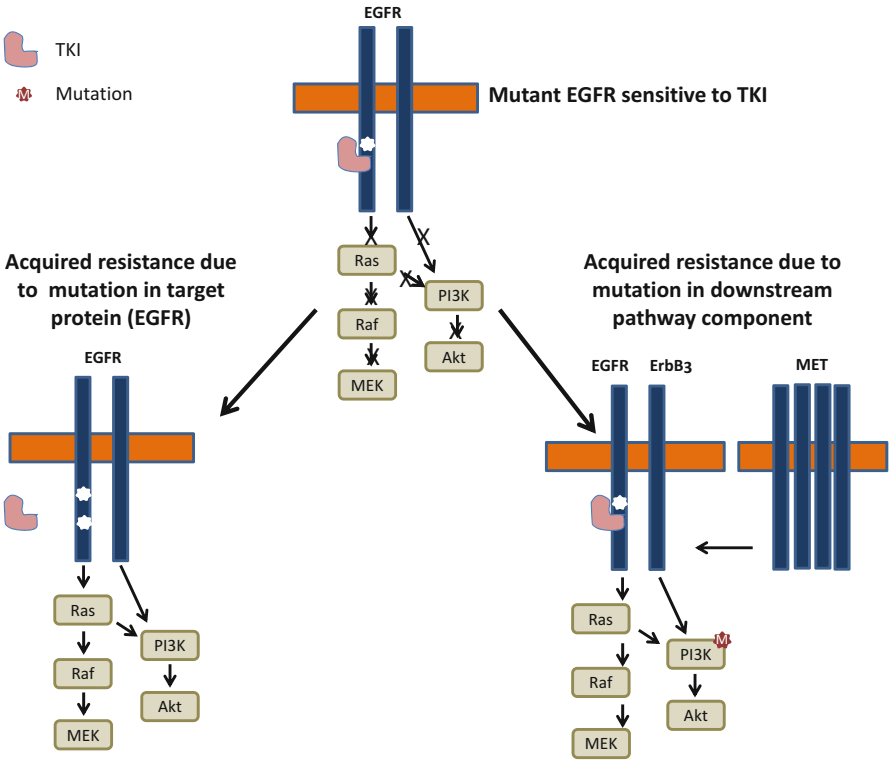

Fig. 12.5 Mechanism of acquired resistance in human cancer

protein of the addicted pathway leads to resistance. Further studies reported acquired resistance in several other targeted therapies, calling it as unified limitation of targeted therapy (Fig. 12.5).

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Part IV
Targeted Cancer Therapy

Chapter 13
The Potential of Targeting Splicing for Cancer Therapy

Ana Rita Grosso and Maria Carmo-Fonseca

Abstract Many molecules currently used to treat cancer patients target proteins encoded by transcripts that are alternatively spliced. As a consequence, the treatment may simultaneously block isoforms with different and sometimes opposing biological activity, thus reducing its efficacy. Recent studies highlight the role of splicing regulation in cancer progression and the importance of the splicing machinery as a therapeutic target. In reviewing this emerging field of cancer biology, we describe very exciting novel findings that illustrate the range of scenarios in which alternative splicing can contribute to all cancer hallmarks, from avoidance of apoptosis to angiogenesis, invasion and acquired resistance to drug therapy. Finally, we address cancer-selective approaches that are being developed to interfere with the splicing machinery and modulate splicing decisions.

Keywords Alternative splicing • Splicing factors • Cancer biology • Cancer therapy • Drug targets

13.1 Introduction

Although cancer is a genetic disease, no single gene defect causes a tumor. Rather, it is only when several genes are altered that cancer arises. Moreover, cancer evolves through successive genetic changes that become advantageous to a cell. In essence, defective genes responsible for tumorigenesis belong to three groups: oncogenes, tumor-suppressor genes and genome stability genes [1]. Defects in oncogenes

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render the gene constitutively active or active under conditions in which the normal gene is not, whereas tumor-suppressor and stability genes become inactivated. These defects in gene function can result from chromosomal translocations, deletions or insertions, amplifications or intragenic mutations. An additional, recently recognized mechanism of oncogene activation or tumor-suppressor and genome stability gene inactivation is alternative splicing. Most human genes produce multiple mRNA isoforms through alternative splicing, and altered splicing is a major contributor to cancer progression. This chapter focuses on the role of alternative splicing in cancer and highlights the therapeutic potential of targeting and modulating cancer-specific splicing isoforms.

13.2 Pre-mRNA Splicing and Its Regulation

RNA splicing was discovered in 1977 as a new mechanism for the biosynthesis of adenovirus mRNA in mammalian cells [2, 3]. Shortly after, cellular genes were also shown to be split into exons and introns. The first examples included the globin [4, 5], the ovalbumin [6] and the immunoglobulin [7] genes. Next, it was recognized that at each intron boundary there are consensus sequences common to vertebrate, plant and yeast cells, suggesting the splicing process was evolutionary conserved [8, 9].

Today we know that the vast majority of human protein-coding genes contain up to 90 % of non-coding sequence in the form of introns that must be spliced from the primary transcripts synthesized by RNA polymerase II (pre-mRNAs). There are over 200,000 different introns in the human genome, ranging in size from <100 to >700,000 nucleotides (nts), with median intron and exon lengths of 1,800 and 123 nts, respectively.

Excision of introns with single nucleotide precision relies on the spliceosome, one of the largest and most elaborate macromolecular machines in the cell [10]. The building blocks of the spliceosome are uridine-rich small nuclear RNAs (UsnRNAs) packaged as ribonucleoprotein particles (snRNPs) that function in conjunction with over 100 distinct non-snRNP auxiliary proteins [11]. The major spliceosomal small nuclear ribonucleoprotein particles comprise the U1, U2, U4, U5 and U6 snRNAs. In addition, human cells have a minor variant form of the spliceosome responsible for excision of about 800 so-called U12-dependent introns that are characterized by a distinct set of splice-site sequences [12–14]. Much of our current understanding of the role of snRNPs in splicing was triggered by studies using human autoantibodies from patients with systemic lupus erythematosus that selectively react with the spliceosomal RNA-protein complexes [15].

The spliceosomal snRNAs recognize, through base pairing, four short consensus sequences termed the exon-intron junctions (5′ splice site and 3′ splice site), the branch point sequence, and the polypyrimidine tract (Fig. 13.1a). Spliceosomes build anew on every intron that is synthesized and then disassemble for the next round of splicing (Fig. 13.1b–e). Assembly of the spliceosome starts with the ATP-independent binding of the U1 snRNP through base-pairing interactions of the 5′ end of the U1 snRNA to the 5′ splice site of the intron. This is followed by the binding of the SF1 protein and the heterodimeric U2 auxiliary factor (U2AF) to the

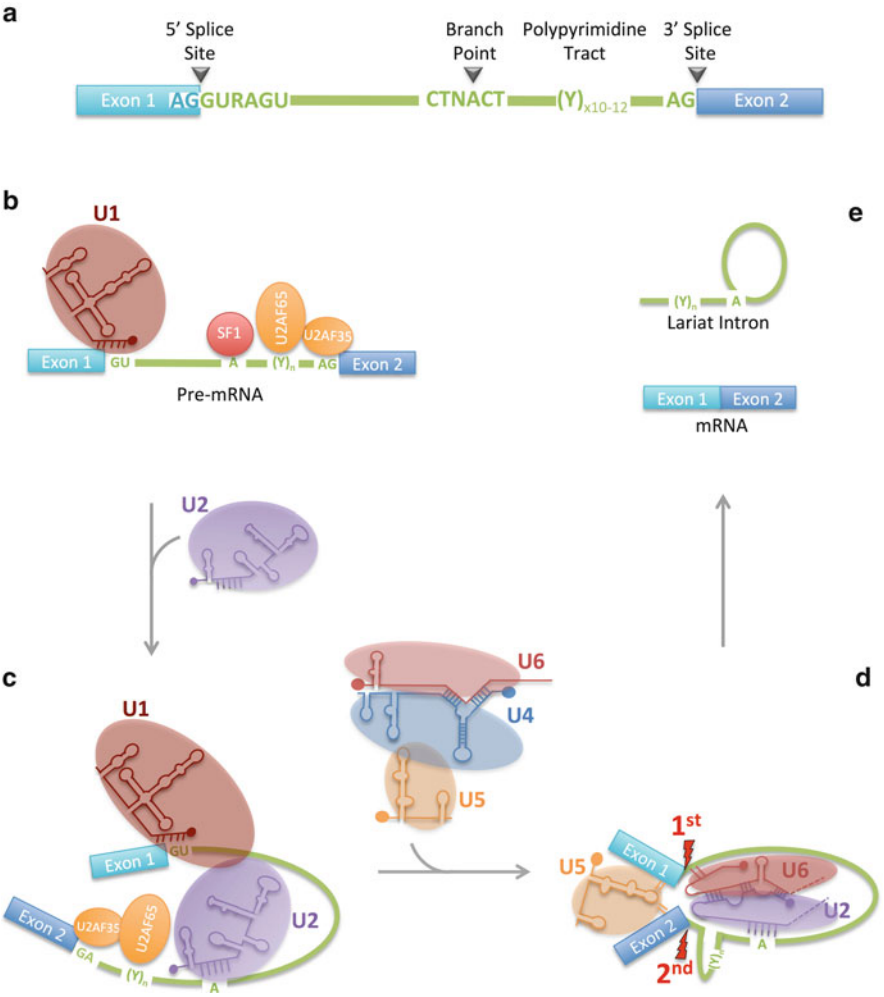

Fig. 13.1 Intron removal by splicing. (**a**) In humans, most introns are removed by the major spliceosome that recognizes conserved sequence elements located at the 5′ splice site, branch point (BP) and 3′ splice site. The polypyrimidine tract is a pyrimidine-rich stretch located between the BP and the 3′ splice site. The panel depicts two exons (*blue*) separated by an intron (*green*). N represents any nucleotide, R a purine, and Y a pyrimidine. (**b**) Spliceosome assembly initiates by binding of the U1 snRNP to the 5′ splice site and recruitment of the U2AF65/U2AF35 heterodimer to the 3′ splice site. The U2AF65 subunit binds to the polypyrimidine tract, the U2AF35 subunit to the AG dinucleotide at the 3′ splice site, and the SF1 protein binds to the branch point sequence. (**c**) In an ATP-dependent reaction, the U2 snRNP displaces SF1 and binds to the branch point. At this stage, the 5′ splice site, branch point sequence, and 3′ splice site, are in close spatial proximity. Bending of the polypyrimidine tract induced by interaction with U2AF brings the 3′ splice site into juxtaposition with the branch point sequence. Both the 5′ and 3′ splice sites are close to the 5′-end of the U2 snRNA, which later assembles with U6 snRNA forming the catalytic center of the spliceosome. (**d**) Catalytic activation occurs subsequent to addition of the U4/U6.U5 tri-snRNP and requires several rearrangements, including departure of the U1 and U4 snRNPs. The splicing reaction consists of two consecutive transesterification (replacement of one phosphodiester linkage for another) events. (**e**) After the two chemical steps of splicing are complete, the spliced exons are released, the spliceosome disassembles and the excised intron is degraded

branch point sequence and the downstream polypyrimidine tract, respectively. These proteins bind cooperatively, with SF1 interacting with the large subunit of U2AF (U2AF65), whereas the small subunit (U2AF35) binds the AG dinucleotide of the 3′ splice site (Fig. 13.1b). Next, the U2 snRNA engages in an ATP-dependent base-pairing interaction with the branch point sequence, displacing SF1 (Fig. 13.1c). Subsequently, the U4, U5 and U6snRNPs are recruited as a preassembled U4/U6.U5 tri-snRNP. With all snRNPs present, the spliceosome undergoes major conformational rearrangements that lead to release of U1 and U4. The spliceosome is now competent to catalyze the first transesterification step of splicing (Fig. 13.1d): the phosphodiester bond at the 5′ splice site is attacked by the 2′-hydroxyl of the adenosine at the branch point sequence, generating a free 5′ exon and an intron lariat-3′ exon intermediate. After additional rearrangements, the spliceosome catalyzes the second transesterification reaction: the 3′-hydroxyl of the 5′ exon attacks the phosphodiester bond at the 3′ splice site, leading to exon ligation and excision of the lariat intron. Then the spliceosome dissociates, releasing the mRNA (Fig. 13.1e).

Most of the functionally important RNA-RNA interactions formed within the spliceosome are weak and require the assistance of auxiliary proteins that bind weakly to specific sequences in exons and introns. This combination of multiple weak interactions is crucial for the flexibility of the spliceosome, in particular during regulated splicing decisions. Recently, fluorescence microscopy has been used to follow assembly of individual yeast spliceosomes in real time. The results indicate that spliceosomal components associate with pre-mRNA sequentially, but each step in the assembly pathway is reversible [16]. This implies that potentially any step during spliceosome formation might be subject to regulation. Spliceosome assembly is indeed highly regulated: depending on the combinatorial effect of proteins that either promote or repress the recognition of the core splicing sequences, splice sites in pre-mRNA can be differentially selected to produce multiple mRNA isoforms (Fig. 13.2). This process is called alternative splicing.

13.3 The Importance of Alternative Splicing

Diverse forms of mRNA are created by the differential use of splice sites (reviewed in [17]). Exons that are always included in the mRNA are called constitutive exons, and exons that are sometimes included and sometimes excluded from the mRNA are called cassette exons (Fig. 13.3). Some pre-mRNAs contain multiple cassette exons that are mutually exclusive, producing mRNAs that always include one of several possible exon choices. Exons can also be lengthened or shortened by altering the position of one of their splice sites alternative 5′ and alternative 3′ splice site selection; (Fig. 13.3). The 5′ and 3′-terminal exons can further be switched by combining alternative splicing with the use of alternative promoters or alternative polyadenylation sites, respectively (Fig. 13.3). Finally, certain intronic sequences may persist in the final mRNA, a splicing pattern called intron retention (Fig. 13.3).

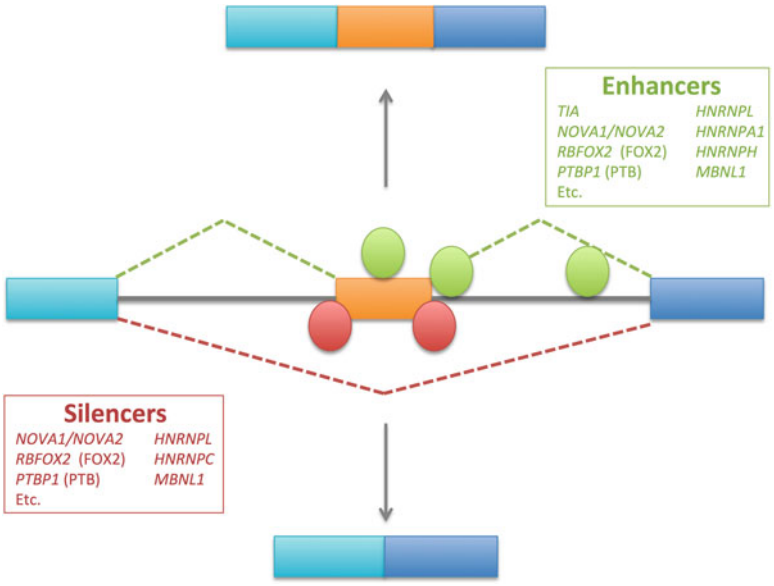

Fig. 13.2 Splicing is regulated by positional binding of RNA-binding proteins. The diagram depicts a model for mechanism of splicing activation or repression by RNA-binding proteins (RBPs). Two constitutively spliced exons (*blue*) are separated by an alternative or cassette exon (*orange*). Pending on the positions at which RBPs bind to the pre-mRNA, the alternative exon is either included (*top*) or excluded (*bottom*). Certain RBPs (depicted *red*) bind at intronic positions close to the 3′ and 5′ splice sites of the alternative exon to silence its inclusion. In contrast, binding of enhancer RBPs (*depicted green*) within the exon or in the downstream intron promotes inclusion of the alternative exon

Alternative splicing was first reported in 1980, when it was discovered that membrane-bound and secreted antibodies are encoded by the same gene [18, 19]. During the 1980s and 1990s many biologically important alternative splicing events were identified and characterized. Yet, the prevalence and general importance of this process was far from clear. More recently, the application of genome-wide profiling technologies coupled with bioinformatic approaches resulted in major advances in our understanding of alternative splicing. In particular, high-throughput massively parallel short-read sequencing provided for the first time unambiguous and unbiased detection of expressed RNA sequences. Compared to microarray-based systems for profiling alternative splicing, short-read sequencing offers a more accurate method for quantifying relative levels of different transcripts. Analysis of human tissue RNA sequencing (RNA-Seq) data revealed that approximately 95 % of human pre-mRNAs that contain more than one exon are spliced to yield multiple mRNAS, and that most isoforms display variable expression across tissues [20, 21]. Genes with few exons typically encode a small number of mRNA isoforms, while tremendously diverse mRNA repertoires can be produced from genes containing numerous exons. For example, the human gene *UTY* has 61 exons and can generate 129 mRNA isoforms (according to UCSC Known Gene annotations [22]).

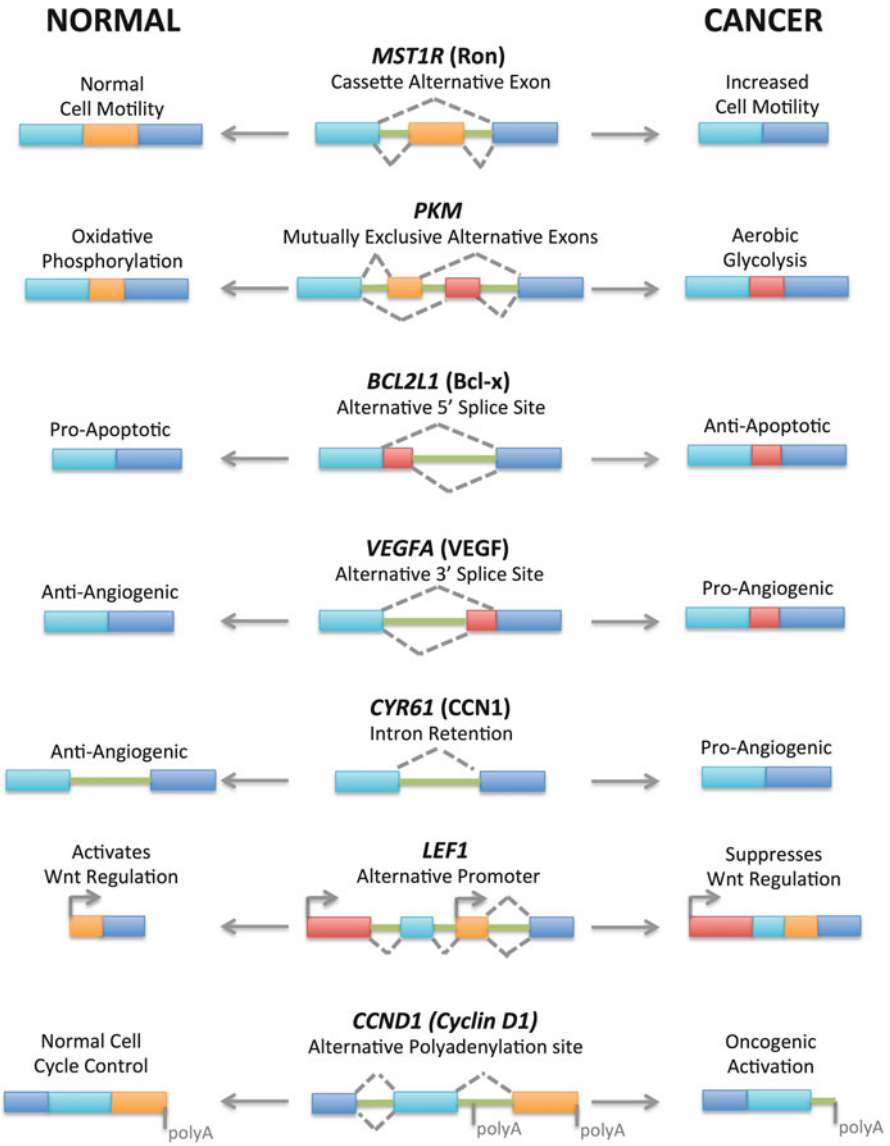

Fig. 13.3 Alternative splicing events in cancer. The basic types of alternative splicing include cassette-exon inclusion or exclusion, alternative 5′ or 3′ splice site selection, intron retention, alternative selection of transcription initiation (alternative promoter) and alternative selection of 3′ end processing sites (alternative polyadenylation). Alternative splicing events that have positive effects on cancer progression are shown. Constitutively spliced exons are depicted in *blue*

Overall, the high prevalence of alternative splicing combined with the finding that many mRNA isoforms represent low-abundant, non-conserved transcripts argue that they may be devoid of functional impact. Yet, recent studies are revealing a rapidly growing number of physiologically important splicing events.

Distinct splicing patterns of a given pre-mRNA can be observed pending on the cellular environment. For example, some mRNA isoforms are specifically expressed in certain tissues or developmental stages and other are triggered in response to external stimuli such as signaling pathways (reviewed in [23, 24]) or depolarization of neurons [25]. The diversity of mRNA isoforms appears to be higher in embryonic stem cells compared to differentiated cells [26] and some isoforms specifically detected in embryonic stem cells have recently been shown to play a key role in pluripotency [27–30]. Remarkably, a single splicing event can function to control an entire transcriptional network. This is well illustrated by alternative splicing of the transcription factor *FOXP1*. *FOXP1* mRNAs transcribed in embryonic stem cells contain a specific exon that is skipped in differentiated cells [30]. Inclusion of this exon determines the DNA binding properties of the encoded protein and is required for stimulating the expression of several pluripotency transcription factor genes [30].

Presumably, the intron-exon structure of genes played an important role in the generation of new genes during evolution. Moreover, alternative splicing seems to be rapidly evolving, particularly among physiologically equivalent organs from vertebrate species [31]. A remarkable example of how species-specific alternative splicing evolved was found in bats [32]. In order to detect warm-blooded prey, vampire bats express a splice isoform of the transient receptor potential cation channel V1 (*TRPV1*) gene. This isoform produces a channel with a truncated carboxy-terminal cytoplasmic domain capable of detecting infrared radiation. In contrast, the protein isoform expressed in fruit-feeding bats has a much higher thermal activation threshold [32].

Around 10–30 % of all alternatively spliced exons have inclusion levels that differ across tissues and are therefore called tissue-specific exons. Many of these exons play an important role in cell differentiation, for example during brain and heart development [33]. Recent large-scale computational analysis revealed that genes with tissue-specific exons tend to have more interaction partners compared to the other genes [34, 35]. Tissue-specific exons tend to encode flexible protein segments without a well-defined three-dimensional structure that likely form conserved interaction surfaces [34, 35]. Using a high-through put coimmunoprecipitation assay, inclusion of tissue-specific exons was shown to both promote and disrupt partner interactions [35]. Thus, tissue-specific splicing can potentially mediate new molecular interactions in a cell type-specific manner.

How human cells control more than 100,000 alternative splicing decisions remains incompletely understood. Clearly, there are multiple mechanisms involved, including RNA-binding proteins that interact with pre-mRNAs and modulate the efficiency of splice-site recognition by the spliceosome, formation of secondary structures in the RNA, the transcription rate and epigenetic modification of the template chromatin [36, 37]. A relatively small number of splicing regulators has been identified, and most are ubiquitously expressed although their relative abundances can fluctuate in different tissues [38]. A few, however, are tissue-specific

RNA-binding proteins, such as *NOVA1* and *NOVA2* [39], *PTBP2* (nPTB) [40, 41], *RBFOX1* (FOX-1) and *RBFOX2* (FOX-2) [42, 43], *ESRP1* and *ESRP2* [44] and *SRRM4* (nSR100) [45]. Currently, large-scale quantification of alternative splicing has been combined with genome-wide identification of in vivo binding sites of splicing regulators (Fig. 13.2) to create maps identifying all pre-mRNAs regulated by a single RNA-binding protein. RNA splicing maps are providing an unprecedented view of the global principles guiding splicing regulation [46].

13.4 Numerous Alterations in Splicing Occur in Cancer Cells

Recent high-throughput transcriptome sequencing studies revealed that different splicing variants are commonly found in cancer tissue compared to the normal surrounding tissue. This type of information has already proven useful in the classification of ovarian and breast cancer [47, 48]. In the case of prostate cancer, alternative splicing signatures are more reliable for diagnostic purposes than are gene expression signatures [49], and in osteosarcoma, changes in relative expression of splicing isoforms of the *TP53* (p53) inhibitor *MDM2* (HDMX) is a more effective prognostic biomarker than *TP53* mutation [50].

Direct causes of splicing alterations in cancer can be grouped into two main categories: *cis*-acting mutations in the pre-mRNA sequence and *trans*-acting changes in expression or activity of regulatory splicing factors. The first category encompasses mutations or polymorphisms in splice sites or regulatory sequence motifs. For example, in breast and ovarian cancer, mutations in the tumor suppressor breast cancer 1, early onset (*BRCA1*) often disrupt constitutive splice sites, leading to the production of inactive protein isoforms [51]. Splice site mutations in the *TP53* gene have also been described in various cancers [52]. In infant B-precursor leukemia, intronic mutations were found in the *CD22* gene that affect target motifs for splicing factors *HNRNPL* (hnRNP-L), *PTBP1* (PTB) and *PCBP1* leading to deletion of exon 12 and expression of a truncated and functionally defective *CD22* coreceptor protein unable to transmit apoptotic signals [53]. Recent systematic surveys identified 106 acquired somatic splice site mutations associated with aberrant splicing in lung cancer [54] and 158 essential splice site mutations in breast cancer [55].

In addition to inherited and acquired mutations, the human genome contains approximately 1,200 single nucleotide polymorphisms (SNPs) expected to modify splicing decisions [56]. A subset of these splicing-related SNPs may be functionally relevant in the context of cancer susceptibility and cancer progression, as shown by a recent study that identified a splicing polymorphism in the germline as predictor of response to targeted therapies [57]. A common intronic deletion polymorphism in the *BCL2L11* (BCL2-like 11, BIM) gene switches splicing from exon 4 to exon 3, leading to expression of *BCL2L11* isoforms that lack the pro-apoptotic BCL2-homology domain 3. The presence of this polymorphism explains why some individuals with chronic myeloid leukemia and epidermal growth factor receptor-mutated non-small-cell lung cancer have inferior responses to tyrosine kinase inhibitors [57].

Changes in expression or activity of *trans*-acting protein factors are caused by defects in components of the spliceosome or splicing regulatory factors. These can be induced by either genetic mutations and amplifications, or transcriptional and post-transcriptional mis-regulation. For example, *SRSF1* (SF2/ASF), a member of the SR protein family of splicing regulators, is frequently upregulated in many cancers. In some tumors, the gene locus is amplified accounting for the elevated levels of the protein [58]. Altered transcriptional regulation by *MYC* (c-Myc), which binds directly to E-boxes in the *SRSF1* gene promoter, is an alternative cause for *SRSF1* protein over expression in cancer [59]. *MYC* can also control expression of additional splicing regulators, namely hnRNP proteins [60]. In addition to *MYC*, other transcription factors control the expression of splicing proteins. Namely, mutations in the Wilms's tumor suppressor gene, *WT1*, abrogates binding of the *WT1* protein to the *SRPK1* promoter, causing over-expression of this SR-protein kinase and hyperphosphorylation of splicing regulator *SRSF1*; this in turn resulted in altered splicing of *VEGFA*, stimulating angiogenesis [61]. Direct binding of the transcription repressor Snail to the *ESRP1* promoter was also shown to cause reduced expression of this epithelial-specific splicing factor, thus promoting isoform switching of several genes involved in epithelial-to-mesenchymal transition [62]. Recurrent mutations in genes encoding essential components of the splicing machinery such as *U2AF1*, *ZRSR2*, *SRSF2*, *SF3A1* and *SF3B1* were recently found in patients with myelodysplastic syndromes [63–66]. More recently, mutations in *U2AF1*, *U2AF2*, and *SF3B1* genes were also detected in lung cancer patients [67]. Remarkably, most of these mutations affect proteins involved in 3′-splice site recognition during the early stages of spliceosome assembly (Fig. 13.1b, c). This, together with the finding that mutations were detected in a mutually exclusive manner, strongly suggests that the compromised function of early spliceosome complexes is a hallmark of cancer.

In general, factors involved in splicing regulation are RNA binding proteins that interact with particular sequence motifs, albeit with relatively low specificity. Therefore, most alternative splicing decisions are controlled by the cooperative binding of several protein factors to short redundant RNA motifs. As a consequence, each alternative splicing event is frequently regulated by multiple factors, and each factor may control several splicing events [46]. A striking example was recently reported for the tissue-specific splicing factors *RBFOX1* (FOX-1) and *RBFOX2* (FOX-2). Unlike most other known splicing regulators, the FOX proteins bind exclusively two defined sequence motifs: UGCAUG and AGCAUG [68]. The expression of *RBFOX2* was found downregulated in ovarian cancer and *RBFOX2* binding sites were detected downstream of one-third of the exons alternatively spliced in this type of cancer [69]. Importantly, reducing the expression of *RBFOX2* in cell lines recapitulated the cancer-associated splicing signature, suggesting that the reduced level of *RBFOX2* causes the changes in splicing [69]. This study further showed that ovarian and breast cancers share a common splicing signature. Although *RBFOX2* transcripts were not downregulated in breast cancer, they were alternatively spliced producing an inactive form of the protein [69]. This illustrates how changes in splicing of a splicing factor can change its regulatory activity, leading to further changes in splicing of its target pre-mRNAs.

Although splicing is primarily controlled by sequence elements in the pre-mRNA that recruit trans-acting splicing factors, recent work make it clear that alternative splicing is also sensitive to transcriptional rate and chromatin conformation (reviewed in [70]). Since many proteins that control DNA and histone modification show aberrant expression or altered activity in tumors [71], it is likely that epigenetics represents a third cause of splicing abnormalities in cancer. Of note in this regard, variations in the methylated status of the *MST1R* (RON) promoter correlate with transcription of a short isoform of the enzyme that is constitutively active and drives cell proliferation [72].

While the majority of cancer-specific alternative splicing events may have just coincidently occurred during tumor development, a few bestow a growth advantage on the tumor. To date, several splicing isoforms that are specifically expressed in cancer have been demonstrated to contribute to cellular malignant phenotypes such as avoidance of apoptosis, angiogenesis, limitless replication potential, and invasion [73], as detailed below (see also Table 13.1 and Fig. 13.3).

13.4.1 Apoptosis

Transcripts from numerous genes involved in apoptosis are alternatively spliced, often resulting in isoforms with opposing roles in promoting or preventing cell death. Well-characterized examples include the *BCL2L1* (Bcl-x), *CASP2* (Caspase-2), *CASP9* (Caspase-9), and *FAS* (Fas) genes. In general, isoforms that enhance survival tend to be up-regulated in cancer and correlated with clinical staging (reviewed in [73]). Another protein that promotes apoptosis upon DNA damage is *AIMP2* (Aminoacyl-tRNA synthetase-interacting multifunctional protein 2). A splicing variant of *AIMP2* was found highly expressed in lung cancer, leading to increased resistance to cell death, and the relative expression of this isoform correlated with cancer stage and survival of patients [75].

13.4.2 Angiogenesis

Primary transcripts encoding Vascular Endothelial Growth Factor (*VEGFA*), which plays a key role in promoting the formation of new blood vessels, undergo extensive alternative splicing. As a result, two families of splicing isoforms are produced with either pro-angiogenic or anti-angiogenic functions. Anti-angiogenic isoforms are expressed in normal tissues and are downregulated in many cancers (reviewed in [124, 125]). Another protein involved in angiogenesis that is regulated by alternative splicing is *CYR61* (cysteine rich 61, CCN1). While normal cells express a variant with retention of an intron that most likely targets the transcripts for degradation, in cancer cells the intron is spliced leading to an accumulation of active protein [85].

Table 13.1 Alternative splicing (AS) events that favor cancer progression

	Gene	AS event type	Altered regulatory proteins	Functional implications	References
DNA damage	*ABL1*	Cassette exon	*EWSR1* (EWS)	Response to DNA damage	[74]
	CHEK2	Cassette exon	*EWSR1* (EWS)	Response to DNA damage	[74]
	MAP4K2	Cassette exon	*EWSR1* (EWS)	Response to DNA damage	[74]
Apoptosis	*AIMP2*	Cassette exon	–	Inhibition of apoptosis	[75]
	BCL2L1 (Bcl-x)	Alternative 5′ splice site	–	Inhibition of apoptosis	[76]
	BCL2L11 (BIM)	Cassette exon	*SRSF1* (ASF/SF2)	Inhibition of apoptosis	[77]
	CASP2 (Caspase-2)	Cassette exon	–	Inhibition of apoptosis	[78]
	CASP9 (Caspase-9)	Cassette exon	*HNRNPL* (hnRNP L)	Inhibition of apoptosis	[79]
	CD22	Cassette exon	*HNRNPL*, *PTBP1*(*PTB*), *PCBP1* (PCB)	Inhibition of apoptosis	[53]
	CFLAR (FLIP)	Cassette exon	–	Inhibition of apoptosis	[80]
	FAS	Cassette exon	*TIA1*, *PTBP1*	Inhibition of apoptosis	[81]
	STAT3	Alternative 3′ splice site	–	Inhibition of apoptosis	[82]
	TP53	Alternative promoter	–	Inhibition of apoptosis	[83]
	TP73	Alternative promoter	–	Inhibition of apoptosis	[84]
Angiogenesis	*CYR61*	Intron retention	–	Inhibition of angiogenesis	[85]
	FLT1 (VEGFR1)	Intron retention	–	Inhibition of angiogenesis	[86]
	KDR (VEGFR2)	Alternative polyadenylation	–	Inhibition of angiogenesis	[87]
	VEGFA	Alternative 3′ splice site	*SRSF1*	Activation of angiogenesis	[88]
Proliferative potential	*CCND1* (Cyclin D1)	Alternative polyadenylation	*EWSR1*	Oncogenic activation	[89]
	CCND1 (Cyclin D1)	Alternative polyadenylation	*SRSF1*	Oncogenic activation	[90]
	CD44	Cassette exon	*ESRP1*	Evasion from oxidative stress	[91]
	CYP19A1	Alternative promoter	–	Activation of tumor growth	[92]
	ERBB2 (HER2)	Cassette exon	–	Activation of tumor growth	[93]
	ERBB4	Cassette exon	–	Activation of tumor growth	[94]
	FYN	Mutually exclusive exons	–	Activation of tumor growth	[95]
	H2AFY (macroH2A.1)	Mutually exclusive exons	*QKI*	Activation of tumor growth	[96]

(continued)

Table 13.1 (continued)

	Gene	AS event type	Altered regulatory proteins	Functional implications	References
	KLF6	Cassette exon	*SRSF1*, *SRSF3*	Activation of tumor growth	[97]
	MDM2	Cassette exon	*EWSR1*	Genotoxic stress response	[98]
	MET	Cassette exon	–	Activation of tumor growth	[99]
	MTOR	Cassette exon	–	Activation of tumor growth	[100]
	LEF1	Alternative promoter	–	Activation of tumor growth	[101]
	PKM	Mutually exclusive exons	*PTBP1*, *HNRNPA1*, *HNRNPA2B1*	Aerobic glycolysis	[60]
	RASSF1	Alternative promoter	–	Activation of tumor growth	[102]
	RPS6KB1 (S6K1)	Cassette exon	*SRSF1*	Oncogenic activation	[58]
	TP63	Alternative promoter	–	Activation of tumor growth	[103]
	TPM1 (tropomyosin)	Intron retention	*DEK*	Activation of tumor growth	[104]
Invasion and metastasis	*ADAM8*	Cassette exon	–	Invasive cell migration	[105]
	CD44	Cassette exon	*SRRM1* (SRm160)	Invasive cell migration	[106]
	CD44	Cassette exon	*ESRP1*	Induction of EMT	[107]
	CPE	Alternative promoter	–	Invasive cell migration	[108]
	CTNND1 (p120)	Alternative promoter	–	Invasive cell migration	[109]
	ENAH (Mena)	Cassette exon	–	Invasive cell migration	[110]
	FGFR2	Mutually exclusive exons	*ESRP1*, *ESRP2*	Induction of EMT	[44]
	GLI1	Cassette exon	–	Invasive cell migration	[111]
	MST1R (Ron)	Cassette exon	*HNRNPA2B1* (hnRNPA2/B1)	Oncogenic activation	[112]
	MST1R (Ron)	Cassette exon	*SRSF1*	Oncogenic activation	[113]
	MST1R (Ron) and *MADD* (IG20)	Cassette exon	*HNRNPH1* (hnRNPH)	Oncogenic activation	[114]
	NCOA3 (AIB1)	Cassette exon	–	Invasive cell migration	[115]
	RAC1	Cassette exon	*SRSF1*, *SRSF3*	Oncogenic activation	[116]
	SENP7	Cassette exon	–	Induction of EMT	[117]
	TP53INP2	Cassette exon	*HNRNPA2B1*	Invasive cell migration	[118]
Cancer therapy	*AR*	Cassette exon	–	Resistance to hormonal therapy	[119]
	BCR	Cassette exon	–	Resistance to drug therapy	[120]
	BRAF	Cassette exon	–	Resistance to drug therapy	[121]
	MKNK2 (MNK2)	Mutually exclusive exons	*SRSF1*	Resistance to drug therapy	[122]
	MS4A1 (CD20)	Cassette exon	–	Resistance to drug therapy	[123]
	TP53 (p53)	Cassette exon	–	Resistance to chemotherapy	[52]

13.4.3 Proliferative Potential

Proliferating cells reprogram their metabolism to engage in aerobic glycolysis (the Warburg effect), in part through alternative splicing of the pre-mRNA that encodes the enzyme pyruvate kinase M, *PKM*. Normal cells express the splicing isoform PKM1, whereas all tumors express PKM2. Importantly, replacing PKM2 with PKM1 in cancer cells reduced tumor growth (reviewed in [126]). Cancer-associated changes in alternative splicing can also result in activation of proto-oncogenes such as *CCND1* (Cyclin D1) [reviewed in 127].

13.4.4 Invasion and Metastasis

A significant reprogramming of alternative splicing occurs during the epithelial-to-mesenchymal transition (EMT), a process by which cancer cells acquire invasive capabilities and become metastatic. EMT-associated changes in splicing affect genes such as *MST1R* (Ron), *RAC1*, *CD44*, *FGFR2*, *CTNND1* (p120-catenin), and *ENAH* (Mena) (reviewed in [128, 129]). Among these, the *CD44* transmembrane protein was one of the first genes for which splicing variants were found associated with metastasis. The expression of specific *CD44* splicing variants correlates with aggressive behavior in several cancer cell types [130–132], and one particular isoform (CD44v8-10) potentiates the ability of cancer cells to defend themselves against reactive oxygen species [91, 133]. Alternative splicing can further contribute to regulate the onset of EMT in cancer cells. Indeed, it was recently found that normal breast epithelia express two splice variants of sentrin/small ubiquitin-like modifier (SUMO)-specific protease 7 (*SENP7*), and breast cancer cells express predominantly the isoform that promotes EMT initiation [117]. Additional examples of alternatively spliced isoforms that promote cancer cell migration and invasion via a gain-of-function mechanism include the truncated glioma-associated oncogene homolog 1, *GLI1* [111, 134], the steroid receptor coactivator 3 (*NCOA3*, AIB1) with a deletion of exon 4, SRC-3Δ4 [115, 135], truncated forms of *ADAM8* (a disintegrin and metalloprotease) [105] and *CPE* (carboxypeptidase E) [108], and alternative inclusion of an exon in the five untranslated region of tumor protein p53 inducible nuclear protein 2 *TP53INP2* [118].

In addition to generating protein isoforms with different biological activities, alternative splicing can also regulate gene expression level through inclusion of premature translation termination codons that target the mRNA for degradation by nonsense-mediated decay [136]. This mechanism causes downregulation of proteins involved in tumor development such as *NFAT5* transcription factor [137] and *CDH1* (E-cadherin) in chronic lymphocytic leukemia [138].

Finally, several lines of recent evidence reveal that splicing contributes for acquired resistance to chemotherapeutic drugs. For example, patients with metastatic melanoma are currently treated with vemurafenib, a newly approved drug that

selectively binds monomers of the most prevalent oncogenic mutation of *BRAF* (B-RAF, V600E), inhibiting its kinase activity. However, most patients acquire resistance within a year of treatment. Different mechanisms have been identified that counteract vemurafenib effectiveness, and one of them consists in expression of truncated forms of the *BRAF* (V600E) protein generated by abnormal pre-mRNA splicing. These splicing isoforms lack the RAS-binding domain and dimerize in a RAS-independent manner therefore rendering the enzyme insensitive to RAF inhibitors [121]. Another example is gemcitabine, the drug used for pancreatic ductal adenocarcinoma. Gemcitabine induces overexpression of splicing factor *SRSF1*, leading to formation of a mitogen activated protein kinase interacting kinase *MKNK2* (MNK2) splicing variant that overrides upstream regulatory pathways and confers resistance to the drug [122]. In chronic myeloid leukemia, one of the mechanisms responsible for resistance to tyrosine kinase inhibitors is the expression of an alternatively spliced *BCR* (BCR-ABL) pre-mRNA that lacks the drug-targeted kinase domain [120, 139] and in B cell malignancies, a splicing isoform of *MS4A1* (CD20) produces a *truncated* protein that loses membrane anchorage and causes resistance to rituximab [123]. Splicing variants of the androgen receptor *AR* may also contribute to the development of castration-resistant prostate cancers [119], and in ovarian cancer expression of a particular *TP53* (p53) splicing isoform correlates with impaired response to primary platinum-based chemotherapy [52].

13.5 Targeting Splicing for Cancer Therapy

In cancer research, much effort is focused on the identification of molecular pathways that are specific to tumor cells and essential for their survival. Cancer-specific splice variants are therefore emerging as highly attractive therapeutic targets, since only cancer cells will be targeted with minimum toxicity towards normal cells. However, in contrast with diagnostic and prognostic purposes for which any discriminating isoform can be a valuable biomarker, the selection of splicing isoforms as drug targets requires detailed functional studies to evaluate their potential in ablating cancer cells. RNA interference (RNAi) screens specifically targeted to silence tumor-associated splicing variants currently represent a valuable tool for identification of isoforms essential for cancer cell survival. Recently, a systematic isoform-specific functional screen of 41 alternatively spliced variants associated with breast and ovarian cancer revealed that targeting the spleen tyrosine kinase *SYK* isoform induced apoptosis, whereas global knockdown of the same gene had no effect [140]. Clearly, the functional contribution of splicing isoforms to tumor behavior should be considered when designing anticancer strategies. This is well illustrated by the limited efficacy of currently used molecules like Bevacizumab that target angiogenesis but do not distinguish between the pro- and the anti-angiogenic splicing isoforms of *VEGFA* [125]. It remains to be studied whether targeting specifically the pro-angiogenic *VEGFA* isoform or treating patients with Bevacizumab only in cases where the anti-angiogenic isoforms are absent will be more beneficial [125].

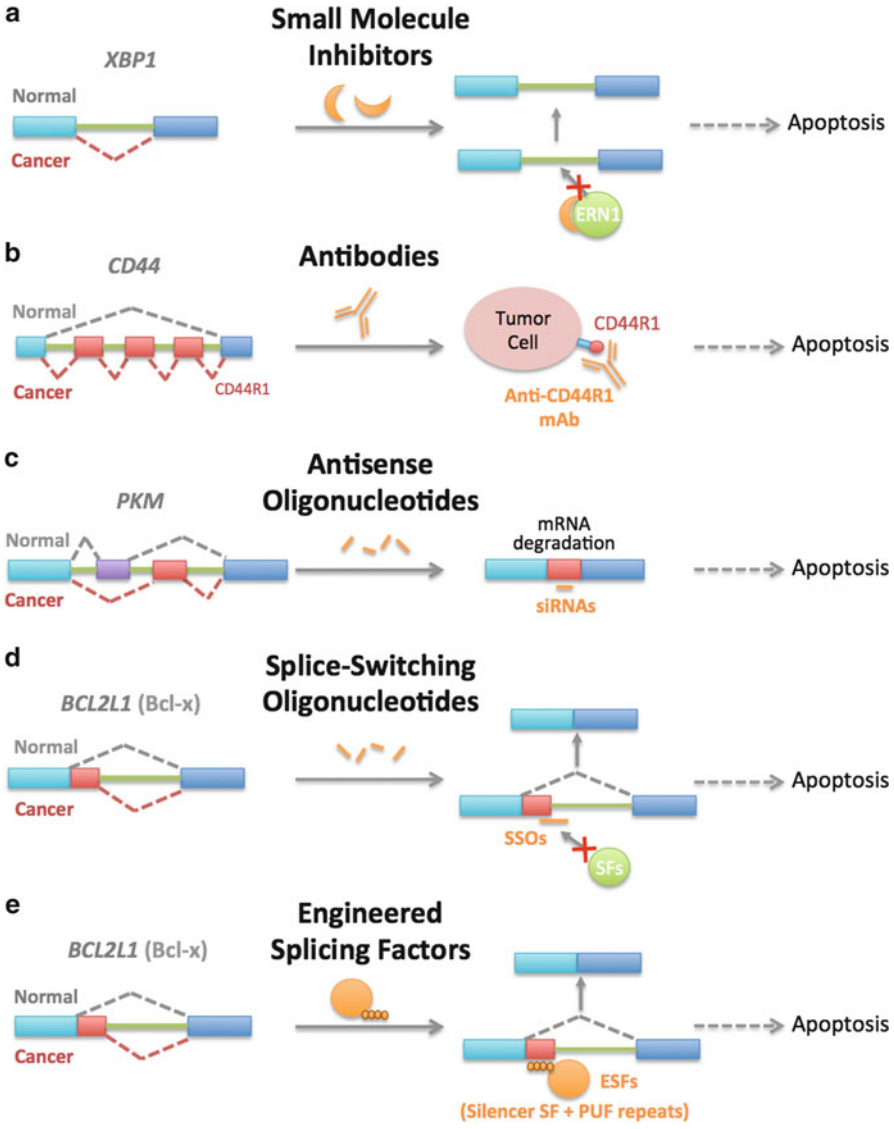

Fig. 13.4 Strategies for splicing-targeted cancer therapies. (**a**) Small molecule inhibitors capable of altering cancer-associated splicing. (**b**) Monoclonal antibodies that recognize unique protein epitopes encoded by cancer-associated mRNA isoforms. (**c**) Antisense oligonucleotides that induce RNAi-mediated knockdown of oncogenic mRNA isoforms. (**d**) Splice-switching oligonucleotides that redirect splicing decisions, thereby reducing oncogenic mRNA isoforms. (**e**) Instead of using oligonucleotides, splicing reprogramming can be achieved by engineered proteins that combine sequence-specific RNA-binding domains with functional domains that regulate splicing

Multiple strategies have been envisioned to therapeutically target cancer-associated splicing. These include small molecule inhibitors, antibodies, and antisense oligonucleotides, as described below in more detail (see Fig. 13.4).

13.5.1 Small Molecule Inhibitors

Different types of molecules capable of altering alternative splicing have emerged from several chemical screens. Many of these substances act by either blocking histone deacetylases or by inhibiting the kinases that phosphorylate SR splicing factors (reviewed in [141]). For example, amiloride can revert cancer-specific splicing events and this effect is likely mediated by changes in amount and phosphorylation status of SR proteins [142, 143]. Similarly, a small-molecule inhibitor of *XBP1* splicing may be a promising therapeutic option in multiple myeloma [144].

Using a different approach, natural products derived from distantly related bacteria were found to target a core component of the spliceosome, the SF3B1 protein, suggesting that interfering with splicing may be a mechanism by which bacteria compete with eukaryotes. Remarkably, mutations in the *SF3B1* gene were found in some cancers [66] and anti-SF3B1 compounds demonstrated dramatic, selective antitumor activity in human tumor xenograft models (reviewed in [145]). The mechanism responsible for such selective antitumor activity is unknown, but one intriguing possibility is that proliferating cancer cells are more vulnerable than normal cells to splicing inhibitors. Consistent with this view, several lines of evidence suggest that RNA splicing is functionally coupled to cell-cycle progression (see [146] and references therein). Moreover, interfering with the splicing machinery leads to activation of *TP53* [147] and induces an alternatively spliced isoform of *TP53* that promotes cellular senescence [148]. Altogether these observations suggest that activation of p53 may contribute to the observed selective anti-tumor activity. Thus, targeting the spliceosome might be a viable approach for development of novel anticancer drugs [145].

13.5.2 Antibodies

An alternative strategy that is being explored consists of raising antibodies against epitopes that are uniquely present in the cancer-associated protein isoforms. A recent example is a fully human monoclonal antibody that recognizes the extracellular domain of a *CD44* isoform expressed on the surface of various epithelial cancers [149].

13.5.3 Antisense Oligonucleotides

Oligonucleotides designed to bind defined sequence elements in the pre-mRNA can induce either an RNAi-mediated specific knockdown of a particular splicing isoform, or redirect splicing decisions. For example, alterations in glucose metabolism mediated by pyruvate kinase (*PKM*) activity are likely to confer a selective advantage for tumor cells to grow in hypoxic environments. Because PK activity is

modulated by alternative splicing, inhibition of the PKM2 isoform that is commonly expressed at high levels in tumor cells appears as a promising target of broad therapeutic applicability. By screening a tiling siRNA library, Goldberg and Sharp recently identified sequences that discriminate between the M1 and M2 splicing isoforms of pyruvate kinase and produce a potent and specific knockdown of the M2 isoform. This resulted in decreased viability and increased apoptosis in multiple cancer cell lines but less so in normal fibroblasts or endothelial cells. Moreover, when the selected siRNAs were delivered as nanoparticles to established xenografts, a substantial reduction of tumor volume was observed [150]. Oligonucleotides can also be designed to redirect splicing decisions through blocking access to the transcript by the spliceosome. Splice-switching oligonucleotides (SSOs) are chemically modified to ensure stability and increase their binding affinity for the target sequence. SSOs have been applied to restore correct splicing of an aberrantly spliced transcript, induce expression of a novel splice variant with therapeutic value, or manipulate alternative splicing from one splice variant to another (reviewed in [151]). The latter mechanism can induce downregulation of a deleterious transcript while simultaneously upregulating expression of a preferred isoform, making it an attractive anti-cancer molecular therapy. Although the application of SSOs is still hindered by poor in vivo delivery to tumor cells, promising results were reported for antisense compounds directed at either inducing the pro-apoptotic splicing variant Bcl-x(S) at the expense of the more abundant survival Bcl-x(L) isoform of *BCL2L1* gene [152], or redirecting splicing of the signal transducer and activator of transcription 3 (*STAT3*) transcripts to produce a truncated isoform lacking the transactivation domain [82]. Enhanced delivery of SSOs to the cell nucleus can be achieved through aptamers that bind nucleolin, a protein that is found on the surface of rapidly proliferating tumor cells and traffics from the cell surface to the nucleus [153].

In principle, splicing can also be modulated using engineered proteins instead of antisense oligonucleotides. A recently proposed strategy relies on direct recognition of the pre-mRNA targets through protein-RNA interaction. Splicing reprogramming is achieved through engineering artificial splicing factors that combine sequence-specific RNA-binding domains with functional domains that regulate splicing [154]. Artificial factors targeted to the human endogenous gene *BCL2L1* (Bcl-X) increased the amount of the pro-apoptotic splicing isoform and promoted apoptosis of cancer cells [154].

13.6 Concluding Remarks

Molecular studies are increasingly used to guide therapeutic decisions for cancer patients, as clinical trials demonstrate superior efficacy of targeted treatments compared to “classical” chemotherapy. Recent advances in high-throughput DNA and RNA sequencing will ultimately lead to a comprehensive characterization of the genome and transcriptome of most cancers. New activating mutations and translocations in oncogenes will be discovered and pursued as drug targets. Additionally, a

more insightful perspective on the contribution of post-transcriptional regulation of gene expression in cancer complexity and diversity will be gained. The complete landscape of splicing alterations will be described for each cancer type and their functional impact on cell growth, metabolism, viability, apoptosis, invasiveness, angiogenesis and drug resistance will be established with the help of RNAi screens. The molecular mechanisms responsible for the functionally relevant cancer-associated splicing events will also be identified. Doubtless, these studies will significantly increase the list of potential cancer therapeutic targets in the near future. The challenge ahead will be to further develop innovative approaches to selectively and efficiently interfere with the splicing machinery and modulate splicing decisions. There is growing optimism that this research area may enable new opportunities for cancer patients.

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Chapter 14
Exploiting Cell Cycle Pathways in Cancer Therapy: New (and Old) Targets and Potential Strategies

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Abstract There is a now a large body of evidence supporting the notion that cancer cells have vastly altered cell cycle networks that serve to maintain their high rate of proliferation. Consequently, targeting these pathways pharmacologically has been long studied, but only recently have some promising compounds progressed into the clinic. In this chapter, we review cell cycle function in both normal cells and describe how cancer cells deregulate this fundamental process. Next we describe in detail the development of different classes of CDK inhibitors and review the failures and successes so far, and provide insight into some future directions for research and clinical trials in order to exploit the ever-expanding molecular characterization of tumors with the drugs available and in the pipelines. In addition, we present a short overview of using differential cell cycle characteristics of normal and tumor cells as a way of protecting normal cells from cytotoxic chemotherapies. Finally we describe other potential targets such as regulating p27, inhibiting PIM and MELK kinases as well as some of the mitotic kinases.

Keywords Cell cycle • Cyclins • CDKs • Synthetic lethality • Mitosis • Combination therapy

14.1 Introduction

Hanahan and Weinberg recognized the importance of cell cycle and checkpoints in their original and updated "hallmarks of cancer" papers, which describe the key features that normal cells must acquire during transformation into a tumor [1, 2].

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Cell cycle deregulation has long been appreciated as a fundamental early event during tumorigenesis, which contributes to several of these hallmarks, namely self-sufficiency in growth signals" and "insensitivity to anti-growth signals", and results in genomic instability, one of the newly added hallmarks. Since these alterations are almost universal among different tumor types, cancer biologists have expended considerable effort in interrogating these pathways as therapeutic targets for 20 years. In spite of the substantial body of literature focused on identifying the biological roles of many cell cycle pathway proteins in both normal development and in describing tumor-associated defects, the progress in the clinic has not been as rapid as desired. With this in mind, we felt that this chapter would be an ideal opportunity for us to review what is known about cell cycle deregulation in cancer, with a focus on personalized treatment strategies. Ultimately, we hope to suggest future directions for research and clinical trials to utilize the wealth of genomic knowledge we now have about cancer, and design more rational strategies likely to be effective in defined genetic contexts, as well as using the cell cycle as a means of protecting normal tissues from the chemotherapeutic insults. We will utilize a particularly promising strategy from our work as an example in a subsequent section.

14.2 Core Cell Cycle Proteins as Targets for Therapy

14.2.1 Cell Cycle Regulation

Some of the best characterized cell cycle targets are key proteins that have been highly conserved throughout evolution in all eucaryotes. These include cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CDKi). Cyclins are master regulators of the cell cycle, via activating CDKs which in turn stimulate downstream signaling (Fig. 14.1).

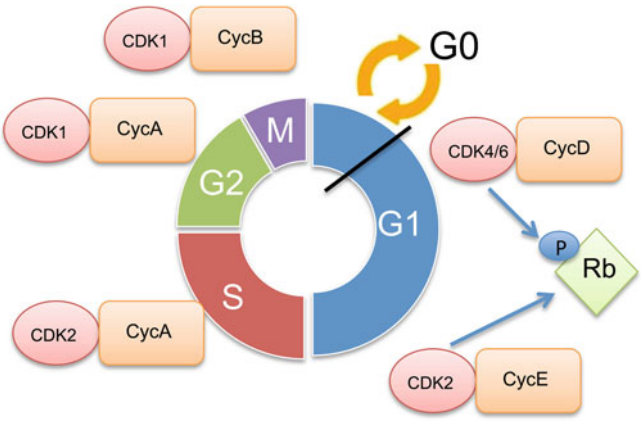

Fig. 14.1 Cell cycle regulation by cyclins and CDKs

Table 14.1 CDK knockout mouse phenotypes

Gene	Phenotype	References
CDK1	Not viable	[7]
CDK2	Sterile but viable	[3, 4]
CDK3	N/A – not active in most mouse strains	[210]
CDK4	Impaired proliferation of pancreatic βcells, leading to diabetes	[5, 211]
CDK6	Hematopoietic deficiency – anemia, thymic development delay	[6]
CDK4 and CDK6	Embryonic lethal between E14.5 and E18.5, severe anemia	[6]
CDK2 and CDK6	Viable but sterile and females are small	[6]

Cell cycle progression is regulated by 4 major families of cyclins, cyclin D, E, A and B, and 4 respective CDKs (CDK4, 6, 2, or 1). Cyclin D is the first cyclin that is involved in the entry of cells from G0 into G1, in response to ample growth factors and other mitogens. Cyclin D exerts its activity via its catalytic partners CDK4 and CDK6, (and can also bind to CDK2 and CDK3) which phosphorylate many substrates. One of the most studied substrates of CDK4/6 is the retinoblastoma protein (Rb), which is a negative regulator of E2F transcriptional activity. Prior to phosphorylation by CDK4/6, Rb is in a hypophosphorylated state, and is bound to E2F and DP proteins, keeping E2F inactive. However CDK4/6 phosphorylation induces conformational change in Rb, releasing E2F to bind DNA and facilitate transcription. The next cyclins that are transcriptionally regulated are cyclin E and A. Cyclin E bound to CDK2 helps drive cells through G1/S transition by further phosphorylating Rb and other substrates involved in DNA replication such as cdc6. Later during S phase, CDK2 is also regulated by cyclin A levels. After DNA replication is complete, cells enter G2 phase where they prepare to enter mitosis by upregulating microtubule formation and other biosynthetic pathways necessary for chromosome segregation. Towards the end of G2 phase, CDK1 takes over as the predominant kinase, since cyclin B levels begin to rise and translocate to the nucleus to bind CDK1 to initiate the G2/M transition. This complex was first identified as the M-phase promoting factor since its main function is to break down the nuclear envelope and initiate prophase. Once mitosis is almost complete, CDK1 is deactivated via dephosphorylation, and a negative feedback loop is engaged via the anaphase-promoting complex which degrades cyclin B, allowing cells to exit mitosis.

The requirement for all of the cyclins and CDKs to control the cell cycle in normal cells as described in the previous paragraph has been recently challenged based on the findings from genetic studies in knockout mice. Tables 14.1 and 14.2 summarize the phenotypes observed in the knockout models. Each interphase CDK has been knocked out individually, and except for CDK1, all of the mice are viable. However, each mouse model has cell-type specific defects, which reveal tissue-specific roles for individual CDKs. For example, the CDK2-deficient model is viable (although born at slightly lower than predicted Mendelian ratio), but sterile due to an absolute requirement for CDK2 during meiosis in both male and female germ cells [3, 4]. Cell cycle analysis and proliferation rate of mouse embryonic fibroblasts from both CDK2 wild-type and knockout embryos showed no significant difference in cell cycle distribution, and a similar rate of proliferation for the first 4 days in culture, after which the knockout cells reached a plateau phase. Similarly

Table 14.2 Cyclin knockout mouse phenotypes

Gene	Phenotype	References
D-type cyclins		
Cyclin D1	Viable, but mice have neurological abnormalities, retinal hypoplasia, decreased body size, and during pregnancy only, impaired mammary epithelial cell proliferation	[212–215]
Cyclin D2	Viable, but females are sterile, and males have decreased sperm counts and hypoplastic testes. Diabetes due to impaired pancreatic beta cell proliferation	[216, 217]
Cyclin D3	Viable, but hypoplastic thymus	[218]
Cyclin D1 and Cyclin D2	Viable until 3 weeks, but decreased body size and hypoplastic cerebellum	[219]
Cyclin D1 and Cyclin D3	Some loss of viability by 4 weeks, most do not survive past 2 months due to respiratory failure and neurological defects	[219]
Cyclin D2 and Cyclin D3	Embryonic lethal at E17.5-E18.5 due to megaloblastic anemia	[219]
Cyclin D1, Cyclin D2 and Cyclin D3	Embryonic lethal at E16.5 due to megaloblastic anemia and defective fetal hematopoiesis	[220]
E-type cyclins		
Cyclin E1	Viable, no detected phenotype	[221, 222]
Cyclin E2	Viable, males are infertile	[221, 222]
Cyclin E1 and Cyclin E2	Embryonic lethal at E11.5. Placental failure due to lack of trophoblast-derived polyploid giant cells in the placenta	[221, 222]
A-type cyclins		
Cyclin A1	Viable, males are infertile	[223, 224]
Cyclin A2	Embryonic lethal at implantation	[223]
B-type cyclins		
Cyclin B1	Embryonic lethal at E10.5, unknown reason	[225]
Cyclin B2	Viable, no detected phenotype	[225]

CDK4 and CDK6 are not necessary for cell cycle progression in most cells, although CDK4 is required for proliferation of pancreatic β-cells, leading to a diabetic phenotype in the knockout mouse [5]. Additionally, CDK6 is important in the hematopoietic system, both in the lymphocytes and erythrocytes. CDK6-deficient mice have small, less cellular thymi, since CDK6 is downstream of Notch and AKT signaling which is critical in early thymic T cell commitment to the T-cell lineage, and also have smaller spleens with less erythroid cells [6]. Double knockout of CDK4 and CDK6 induces late embryonic lethality, primarily due to the hematopoietic defects in erythroid cell production, however MEF cells from these embryos do proliferate and can become immortalized by continuous passage. CDK2 can partially compensate to phosphorylate Rb in these double-knockout cells by binding cyclin D, and therefore promote cell cycle progression, however this is not thought to fully explain the lack of cell cycle defects systemically. In stark contrast to the phenotypes seen in CDK2/4/6 knockout models, CDK1 deficiency causes cell cycle arrest and prevents embryos from developing beyond the 2-cell stage, demonstrating a lack of compensation between the mitotic CDK and the interphase CDKs [7].

Unlike normal cells that do not depend on any single cyclin or CDK for growth, there is beginning to be some evidence that in tumor cells, altered cellular wiring can lead to oncogenic addiction to CDK signaling. For example, in a mouse model of triple negative breast cancer driven by low-molecular weight cyclin E, tumors are highly dependent upon CDK2 signaling, even though CDK2 is dispensable in normal cells [8]. This type of variation on synthetic lethality involving CDK signaling is not limited to breast cancer but can be observed in a K-Ras mutant lung cancer model. In a K-Ras-driven mouse model of lung cancer, CDK2 and CDK6 knockout only partially inhibited tumor initiation, whereas CDK4 knockout significantly decreased tumorigenesis due to an immediate induction of senescence [9]. Even though the K-Ras-mutant transgene is expressed in several other epithelial tissues in this mouse model, none of these undergo hyperplasia or tumorigenesis, and senescence is not observed in these normal tissues. CDK4 was also shown to be essential for progression of established K-Ras driven NSCLC lesions, and pharmacological CDK4 inhibition significantly inhibited tumorigenesis. The reasons for lack of immediate compensation mechanisms involving other CDKs in tumors are not clear, but this phenomenon may allow us to turn this frequent observation into an Achilles heel in cancer cells if we carefully dissect true dependencies in well-planned genetic experiments.

14.2.2 CDK Inhibitors

14.2.2.1 Pan-CDK Inhibitors

Since CDKs are the catalytically active drivers of cell cycle progression, targeting them pharmacologically has been a major effort. The early generation inhibitors, developed more than 15 years ago were pan-inhibitors targeting a large spectrum of CDKs. These drugs were somewhat promising based upon cell line and xenograft work, but when moved into early stage clinical studies failed to show considerable net benefit. The reasons for failure are likely multi-factorial, and include both biological issues as well clinical trial design flaws. In the forthcoming section we will describe the development of several generations of inhibitors and their related trials, and provide insights into future development of these classes of compounds. Figure 14.2 shows the structures of all the CDK inhibitors discussed in this section.

The most extensively tested pan-CDK inhibitor is flavopiridol, which inhibits all four of the interphase CDKs as well as CDK7 and is also the most potent known CDK9 inhibitor [10, 11]. CDK7 is both a cell cycle and a transcriptional CDK, since it is a part of the transcription factor IIH (TFIIH) complex with cyclin H and MAT [12, 13]. CDK7 promotes transcription elongation by phosphorylating the C-terminus of RNA polymerase II. CDK9 is also thought to be involved in transcriptional regulation, in complex with cyclin T, via phosphorylating different sites in the C-terminus of RNA polymerase II [14]. There is a profound response to flavopiridol in cells, that encompasses both cell cycle arrest in G1 and G2, but also transcriptional changes especially in mRNAs with short half lives such as early

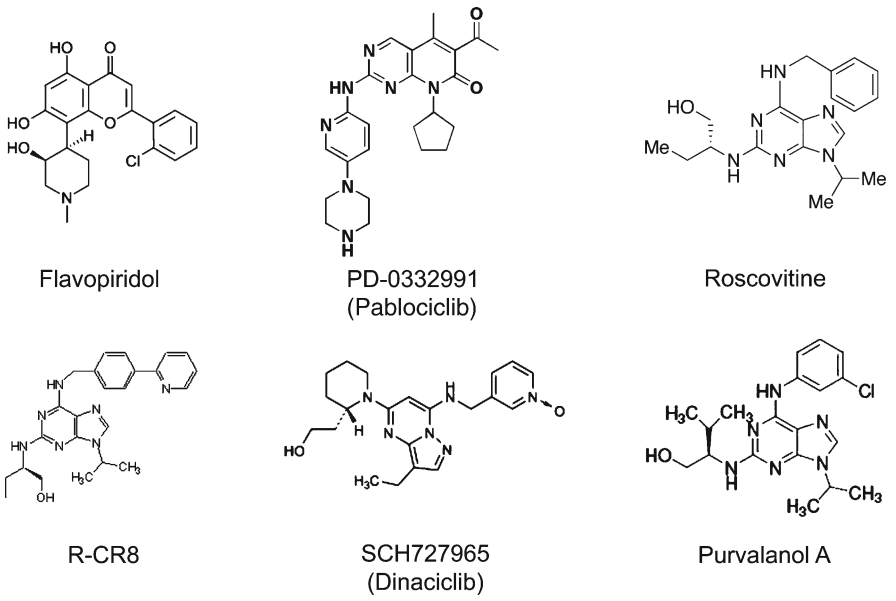

Fig. 14.2 Structures of CDK inhibitors

response transcription factors, apoptosis regulating genes (like Mcl1) and NFκB responsive genes [11, 15, 16]. Whether these responses truly translated when this agent was tested in the clinic was not well studied.

Preclinical data had suggested that prolonged exposure to flavopiridol was necessary for maximal anti-tumor effect, so the two phase 1 trials that opened in 1994 used long infusions (72 h). The dose-limiting toxicities seen were primarily diarrhea, and at higher doses hypotension, anorexia and muscle weakness, and 21 % of patients had venous thromboses. Pharmaokinetic analysis of steady state plasma concentration revealed that 200–400nM was the range reached at the maximum tolerated dose [17]. The prior preclinical studies had found that for maximum activity, a higher concentration in the micromolar range would be desirable, so future studies attempted to reach these levels via bolus dosing on a 1 h per day for five consecutive days schedule. In these later trials, low micromolar peak concentrations were observed, and similar toxicities were observed [18]. However, when several phase 2 studies in solid tumors were analyzed, the enthusiasm for this agent waned, since no objective responses were seen in tumors ranging from melanoma to endometrial carcinoma [19–21]. In contrast, the results seen in hematopoietic malignancies appeared more promising [17]. For example, in chronic lymphocytic leukemia, 40 % of patients had partial responses, and the dose-limiting toxicity observed was tumor-lysis syndrome, indicative of strong anti-tumor activity of this agent [22].

In addition to studies using flavopiridol as a single agent, combination studies were pursued based on the hypothesis that flavopiridol may have benefit as a chemosensitizer. This hypothesis was generated based on the pre-clinical observation that

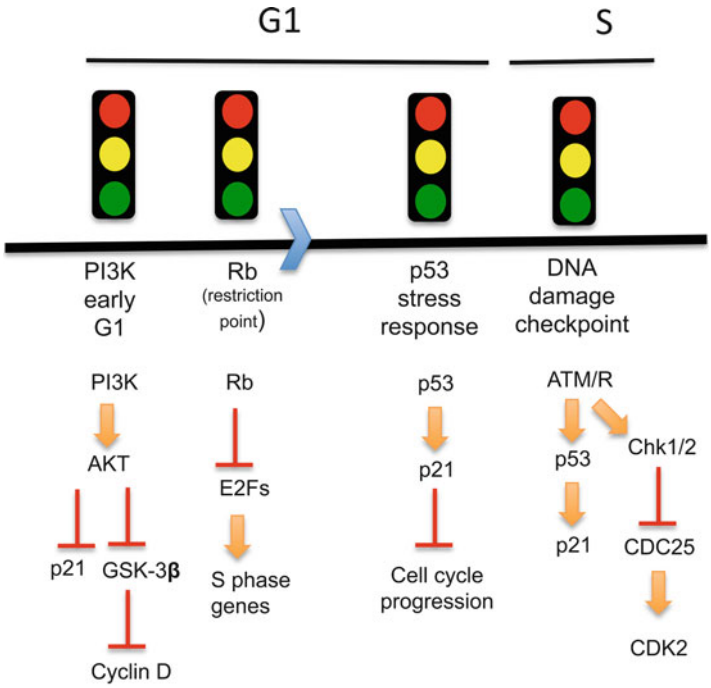

Fig. 14.3 Key regulators of G1 to S phase transition

synchronizing the cells into S phase sensitized them to flavopiridol-induced cytotoxicity, resulting in E2F dependent cell death that is selective to transformed cells [23]. These studies used a variety of classes of cytotoxic drugs including platinum agents, anthracyclines, taxanes and 5-fluorouracil. These studies had more promising results, including a 30–40 % rate of partial responses in some studies [24–31].

In spite of some of these promising activities in both solid tumors and leukemias, recently, there has not been significant progress with this agent. The chemistry of the agent does have some challenges, since it binds to plasma proteins and also is poorly water soluble [32, 33]. There has been a novel liposomal formulation reported a few years ago, which aimed to improve the therapeutic index by slowly releasing the drug to effectively synchronize a large portion of the tumor cell population, while not being bound up in the circulation by plasma proteins [34]. As of writing, there have not been any clinical studies presented or registered using this formulation.

14.2.2.2 Selective CDK4/6 Inhibitors

The G1-S checkpoint (see Fig. 14.3) is altered in close to 90 % of human tumors, by various mechanisms, indicating that this phenotype provides a selective advantage for proliferation and/or survival. With this in mind, selectively targeting CDK4 and

CDK6 has been considered as an alternative strategy in several diseases including breast cancer due to the prevalence of amplification/overexpression of cyclin D1 observed (15–20 % amplification/50–70 % overexpression overall) [35, 36]. One drug candidate, PD-0332991 (see Fig. 14.2 for structure), has quickly moved to the top of its class and has rapidly moved into clinical studies [37]. This compound was selected from a high-throughput screen of pyridopyrimidines, in which both potent anti-proliferative and selective inhibition of CDK4 were used as criteria [38]. When tested against a large panel of other kinases, PD-0332291 (Pablociclib) had a highly selectivity index towards CDK4 and CDK6 (IC50's 11/16nM, versus >8–10 μM for 36 other kinases tested). When tested in MDA-MB435 breast carcinoma cells, PD-0332991 induced a robust G1 arrest, and concomitant reduction of phosphorylation of Rb at the CDK4/6 phosphorylation sites (Ser 780–795). As expected, in Rb-negative cell lines, this compound has no activity, further demonstrating that its mechanism of action includes inhibition of CDK4/6 phosphorylation of Rb [39]. Another marker of resistance that has been identified is elevated p16 expression, since CDK4/6 is physically bound and unable to be inhibited [40].

Breast cancer is the model system which has been best studied so far in terms of understanding mechanism of action, potential synergistic combinations and predictors of resistance. At the molecular level, breast cancers can be divided into luminal or basal based on gene expression signatures. Most of the sensitive cell lines are luminal in nature, and all have intact Rb signaling, whereas the resistant cells tend to be basal-like and lack Rb activity [41, 42]. Intriguingly, basal cell lines, which retain Rb activity are still unresponsive to PD-0332991, and have hyperphosphorylated Rb. It is unclear at present what the precise mechanism is that drives hyperphosphorylation of Rb in these cell lines. It is possible that there is a greater dependence upon CDK2/CDK1, in which case these cells might respond to a combination of CDK2/1 and CDK4/6 inhibitors. Luminal tumors encompass both estrogen-receptor (ER) positive and many HER2-amplified tumors, so naturally combinations of ER antagonists or HER2 inhibiting drugs with CDK4/6 inhibitors were tested. In ER-positive cell lines, treatment with tamoxifen and PD-0332991 resulted in synergism and G1 arrest, and similarly trastuzumab and PD-0332991 are synergistic in HER2-amplified cell lines [41].

Apart from breast cancer cell lines, PD-0332991 has now been evaluated in a variety of other solid tumor types, including pancreatic neuroendocrine tumors, glioblastoma multiforme, rhabdomyosarcoma and mantle cell lymphoma with similar results [43–47]. In xenograft experiments, this drug is mostly cytostatic, with a few examples of cytotoxicity. In addition, PD-0332991 has been explored as a radiosensitizer in glioblastoma, due to its high penetrance of the blood-brain barrier, and preclinically appears to be useful in this scenario [47].

However not all tumor contexts are ideal candidates for such a strategy even if the underlying genetic changes would predict sensitivity. A recent paper described an unanticipated effect observed in pancreatic adenocarcinoma (PDAC). In PDAC cell lines examined, PD-0332991 had anti-proliferative activity, induced robust G1 arrest and hypophosphorylation of Rb [48]. However, gene expression analysis revealed that PD-0332991 upregulated genes involved in pro-angiogenic signaling,

cell adhesion, cell migration/ECM remodeling, and inflammatory pathways. In addition, EMT was induced correlating with increased invasion via TGFβ-SMAD4 signaling, suggesting perhaps combinations of TGFβ inhibitors with CDK4/6 inhibitors might be a way forward in PDAC tumors expressing wild-type SMAD4. Genetic manipulation of CDK4/6 recapitulated this phenotype, ruling out a drug-mediated off-target kinase inhibition.

The first phase 1 study performed using PD-0332991 was recently published, and examined patients with Rb-positive advanced cancers [49]. This study showed that the drug was generally well tolerated, with the main toxicity being myelosuppression, consistent with other cell cycle targeted therapies. Pharmacokinetic analysis suggested favorable properties including slow absorption and elimination. The response rate was moderate (~27 %), however given all the usual caveats of generalizing based on phase 1 studies, the patients who derived some benefit (i.e. stable disease) could tolerate the drug well enough to remain on study for 10+ cycles. In breast cancer patients, a randomized phase I-II study utilizing PD-0332991 in combination with letrozole in ER-positive, HER2-normal post-menopausal patients has been completed (personal communication). In the phase 1 portion, there has been no biomarker selection, but in the phase II portion, the trial is specifically focused on patients with cyclin D1 amplification and/or loss of p16, since these are the patients predicted to respond best. So far, the clinical benefit rate in this combination trial was 70%, which resulted in statistically significant increase in progression-free survival and the adverse event profiles are very similar to what was reported in the single-agent phase 1 studies trials previously discussed i.e. this combination is generally well tolerated. The few patients so far in the study have been safely treated with some patients having partial responses. These well-designed trials with integrated biomarkers built in, are likely to provide more useful information not only about safety and pharmacokinetics but also pharmacogenomics information about responders and the biology behind responses. Caution must be taken though in considering combinations with chemotherapies that depend on actively cycling cells, based on a recent study that showed that PD-0332991 actually protected RB-positive MDA-MB-231 cells from paclitaxel-induced mitotic catastrophe [50]. Pre-treatment with PD-0322991 also resulted in a switch from homologous recombination (HR) DNA repair mechanism to non-homologous end-joining (NHEJ), which is an error prone pathway that could potentially induce further genomic instability in cancer cells. While this finding is intriguing and worthy of further mechanistic study in a broader panel of cell lines and normal mammary epithelial cells, it still remains to be determined how this result will be translated into ER+/HER2+ cell lines, which have been the focus of the majority of the breast cancer studies using this agent.

Going forward in hormone-receptor positive breast cancer, targeting CDK4/6 should eventually become a first line therapy in combination with endocrine therapy for a number of reasons. Certainly, as described above, the preclinical data regarding this combination is compelling, and this includes cell lines that acquired tamoxifen resistance being re-sensitized by PD-0332991. Secondly, genomic data have confirmed the relevance of this pathway in resistance to endocrine therapy alone, such as the fact that cyclin D1 is overexpressed/amplified in endocrine-therapy resistant tumors [51, 52]. In addition to deregulating the cell cycle, amplified cyclin

D can directly activate ER in a hormonally-independent manner that does not require CDK/Rb activity [53]. Thirdly, the fact that cyclin D1-CDK4 is downstream of multiple pathways that mediate resistance to anti-estrogens (e.g. EGFR/HER2, ERK, AKT, NFκB) may make this strategy useful regardless of which pathways are upregulated in any particular patient [54]. Despite being a good target however, resistance to CDK4/6 inhibition is likely to occur, since resistance arises to every targeted therapy tested so far. Indeed, there is evidence currently for activation of CDK2 due to p27 down regulation as a mechanism of resistance to these agents, which could potentially be targeted via CDK2 inhibitors as will be discussed in the next section [55].

Further clinical studies in other diseases are also underway, for example in mantle cell lymphoma. The single agent study showed some evidence of benefit, and now a subsequent study has been designed using PD-0332991 in combination with bortezomib in this patient subset.

14.2.2.3 Selective CDK1/2 Inhibitors

The other subclass of CDK inhibitors that have been developed are more specific for CDK1 and CDK2 (versus CDK4/6), such as R-roscovitine (also known as CYC202 or seliciclib), SNS-032 and the newer agent SCH727965 (see Fig. 14.2 for the structures). Roscovitine, a 2,6,9-trisubstituted purine was generated in a screen of olomoucine-related analogues for CDK1/cyclin B inhibition, and found to potently inhibit CDK1 kinase activity (IC50 of 0.45 μM) [56]. Once olomoucine was shown to co-crystalize with CDK2, roscovitine was also confirmed to bind directly to CDK2 [57]. Several years later, roscovitine became the first orally bioavailable drug from this class to go into clinical trials based on the preclinical data showing that CDK1/2 inhibition causes S and G2 arrest followed by apoptosis in tumor cells [58–60]. Apart from the effects on these cell cycle CDKs, roscovitine also inhibits CDK7 and CDK9, thereby inhibiting transcription as well, via reducing key anti-apoptotic proteins such as Mcl1 [61–64].

Several studies have shown that apoptosis is further induced when CDK1/2 inhibitors are combined with most cytotoxic therapies including taxanes, anthracyclines as well as radiation. For example, a combination of purvalanol A and taxol caused profound apoptosis in Hela cells, when taxol was used first to stabilize microtubules then purvalanol A was added [60]. Intriguingly when the drugs were used in the reverse order, the response was decreased, demonstrating that synchronization of cells in mitosis (i.e. the end results of taxol) is important for the mechanism of CDK inhibitor-induced cell death. A similar synergistic combination strategy was demonstrated in MCF7 breast cancer xenografts using roscovitine and doxorubicin, however in this context, cell cycle synchronization in G2/M phase with roscovitine was used to prime the cells to respond to doxorubicin (versus the taxol→CDKi strategy in Hela cells) [65, 66]. Similar to the Hela cell study described above, the taxol-purvalanol A combination was found to be similarly effective in MCF7 xenografts [67]. These dichotomous results in two different systems just

illustrate one of the challenges we have moving forward with sequential combination therapies that exploit mechanism of action of drugs. Likely a number of factors could contribute to which direction of treatment is likely to be best, including genomic factors (such as Rb status, p53 pathway status), timing of exposure to agents as well as which specific drugs under investigation. Clearly, further mechanistic work is still needed to dissect out these details in order to rationally match treatments to individual patients.

In spite of this incomplete understanding of mechanism of action of these agents in both solid tumors and hematopoietic malignancies, roscovitine was moved into clinical trials in the early 2000s [68]. The phase 1 studies demonstrated that this agent could be administered both in an intravenous formulation as well as orally [69, 70]. It has reasonable pharmacokinetic properties including high bioavailability, slow GI absorption, however it is rapidly metabolized to an inactive metabolite, making its half-life fairly short (~1 h). However, there were a number of dose-limiting side effects observed including liver and kidney toxicity, electrolyte disturbances, rashes and fatigue that accumulated over time, making repeated administration daily for more than 5 days too challenging for patients. Responses were unimpressive over a few phase 1 studies, with primarily stable disease and very few partial responses seen as monotherapy. Two phase 2 studies were undertaken in non-small cell lung cancer and nasopharngeal carcinoma which essentially replicated the results in the phase 1 studies [71]. Looking at the pharmacological and response data together, the researchers concluded that one of the major challenges was maintaining a plasma dose that is high enough for anti-tumor activity based on the preclinical studies, and even when white blood cells were used as a surrogate for tumor cells, Rb phosphorylation was not decreased, further supporting the claim of insufficient dose reaching tumor cells. One way of potentially overcoming this problem might be to use a more frequent dosing schedule such as 2–3 times a day, since the preclinical studies showed that 8–16 h of continued exposure is needed to effectively inhibit tumor growth. Whether this would actually work might not be known since the excitement regarding this agent has waned, in light of newer compounds that have been developed. One such potential compound is CR8, which is a N6-biaryl-substituted derivative of roscovitine that is 2–4 fold more potent at inhibiting CDK1, CDK2 and CDK7, which translated to 40–70 fold higher potency in cellular activity measures such as PARP cleavage and caspase activation [72–74]. Since discovery of this compound a few years ago, animal studies have not been published in cancer models, although a very recent paper utilizing CR8 in a mouse model of traumatic brain injury demonstrated that this drug could be delivered safely *in vivo*.

The other CDK inhibitor that targets CDK1 and CDK2 (as well as CDK5 and CDK9) that appears promising is SCH727965 (Dinaciclib) (see Fig. 14.2 for structure), a compound developed to address some of the issues with previous generation inhibitors with respect to therapeutic index [75]. Indeed, in direct comparison to flavopiridol this agent had > tenfold greater therapeutic index in the A2780 ovarian cancer xenograft model (defined as the ratio between MTD defined as 20 % body weight loss and minimal effective dose to cause 50 % inhibition of tumor growth when given i.p. once daily for 7 days). In addition, cell line studies showed that even

a brief 2 h exposure to SCH727965 was sufficient to inhibit progression of cells into S phase. *In vivo*, this drug was at least as effective as paclitaxel in A2780 xenografts, and well tolerated with the main toxicity being reversible myelosuppression.

In addition to ovarian cancer, SCH727965 has been tested and found to be potentially effective in pancreatic cancer, melanoma and various forms of sarcoma including osteosarcoma [76–79]. The pancreatic study was particularly exciting, as it was performed using low-passage patient-derived xenografts (PDX) as opposed to cell line xenografts. These PDX models are thought to more faithfully recapitulate human tumorigenesis for multiple reasons including the fact that they maintain human stroma for multiple passages [80]. This intense desmoplastic stroma and hypovascular microenvironment which characterizes pancreatic cancer, is known to be a major barrier to chemotherapy drug access. Therefore the data showing efficacy in multiple mouse models with these characteristics bodes well for subsequent trials in humans. In addition to using these better disease models, the authors performed gene set enrichment analysis on the tumors to interrogate potential mechanisms of resistance, an area of research that is very undeveloped in the cell cycle field. They compared sensitive and resistant tumors, and found that in the most resistant tumors, the Notch and TGFβ pathways were upregulated, suggesting that perhaps combinations of these inhibitors may be future directions for research.

Preclinical studies in adult and pediatric leukemia are also underway, and a report of efficacy in CLL cells showed promise independent of high-risk genomic features (del 17p13.1 and gVHI unmutated). Short-term exposure of CLL cells directly taken from patients was sufficient to induce apoptosis [81]. Moreover, SCH727965 was shown to abrogate microenvironment-derived cytokine-induced survival signaling, in a PI3K-dependent mechanism, suggesting that a logical combination to explore might be SCH727965 in combination with PI3K inhibitors.

These pre-clinical studies provided strong rationale for moving this agent into clinical studies. A phase 1 study using SCH727965 dosed once every 3 weeks as a single agent in unselected adult patients has been performed which showed moderate responses (mainly stable disease) and similar to the preclinical studies, myelosuppression was the DLT [82]. Notably, unlike flavopiridol, there was no diarrhea, and much less fatigue, making this agent better tolerated. Since nausea and vomiting were common side effects as well, a subset of patients was given the anti-emetic drug aprepitant [83]. Aprepitant is known to weakly inhibit CYP3A4 which is one of the enzymes involved in metabolizing SCH727965, so the pharmacokinetic parameters were compared in patients treated with aprepitant with those not given it. These results showed no interaction between aprepitant and SCH727965, suggesting that use of this agent prophylactically in the clinic is a feasible and safe strategy moving forward to maximize use of SCH727965 in different patient populations.

Several phase 2 studies with SCH727965 as a single agent have also begun in both solid tumors and hematopoietic diseases, and some have been presented as abstracts at meetings. The first and most promising was performed in adult acute myeloid or lymphocytic leukemia patients and reported at ASH in 2010 [84]. The response rate was 60 %, and many patients had rapid decrease in their blast counts. Correlative studies including pharmacodynamics analyses showed that CDK activity

(i.e. Rb phosphorylation and Mcl1 decreased expression) was effectively inhibited in the samples taken at 4 h post infusion, however these biomarkers returned almost to baseline by 24 h, suggesting a need for frequent dosing. A similar study is also underway in multiple myeloma, but no results are currently available [85].

With respect to solid tumors being examined in single-agent phase 2 trials, the progress has been slower. One single arm study has been reported in melanoma, which had ~72 patients enrolled [86]. The response profiles were very modest, with 22 % of patients with stable disease and no partial or complete responses, and toxicities were common. Another phase 1–2 study in unresectable melanoma is now approved and about to open. The only other trial that has been started is a multi-arm randomized phase 2 in breast cancer and NSCLC, in which SCH727965 is being compared to active treatments for each respective disease (oral capecitabine for breast cancer, and erlotinib for NSCLC). Importantly crossover from the control arm was allowed after disease progression, which is likely to make detection of a significant difference in overall survival extremely challenging. The study has been completed but no data has been presented as of writing (Nov 2012) [87].

Similar to the other CDK inhibitors, combinations with chemotherapy/other targeted agents are ultimately going to be necessary for optimal activity of SCH727965, and already a number of combination studies have been started. These trials include combinations with the PARP inhibitor Veliparib with or without carboplatin, rituximab in CLL, bortezomib and dexamethasone in myeloma, and our own trial with epirubicin in triple negative breast cancer [88–91]. Our trial differs from the others in that by limiting our patients to a specific subtype of breast cancer in which we have preclinical data showing dependence on CDK2 signaling because of LMW-E expression [92]. In addition, another group has shown elevated c-Myc expression is synthetically lethal with CDK inhibition in triple negative breast cancer [93]. By pre-selecting patients with a high likelihood of being oncogenically addicted to CDKs either via the LMW-E pathway or via amplification of c-Myc, we believe we will enrich for potential responders. Further biomarkers of response or markers predicting resistance will be needed in order to make this drug clinically useful. For example, basal like breast cancers or serous ovarian cancers with defects in DNA repair such as BRCA1/2 or ATM mutations may be more sensitive to CDK2 inhibition (in combination with chemotherapy), analogous to their propensity for sensitivity to PARP inhibition. BRCA1 or ATM knockdown sensitized various cell lines to CDK2 inhibitors, since CDK2 can regulate DNA repair independently of its effect on the cell cycle [94].

14.2.3 Targeting p27

Moving away from directly targeting components of the cell cycle that promote growth, the other strategy that has been proposed is targeting negative regulators such as p27. p27 is a small nuclear protein involved in negatively regulating G1 to S phase transition via inhibiting cyclin E-CDK2, cyclin A-CDK2 and cyclin D-CDK4/6

activity [95]. However, in addition, it has novel functions when mislocalized in the cytoplasm that contribute to tumor cell survival and cancer progression [95]. For example, in response to stress, p27 is driven into the cytoplasm via AMPK-mediated phosphorylation at Thr198 which blocks apoptosis and induces a cytoprotective autophagy response [96, 97]. Other cytoplasmic functions also include increased invasion and metastatic potential, perhaps via binding to RhoA and stimulating changes in actin cytoskeleton formation [98]. A p27 knock-in mouse model where p27 has been mutated to be unable to bind cyclins and CDK2, was generated to determine whether there are cell-cycle independent roles for p27 [99]. One of the major findings from this model includes a role for cytoplasmic p27 in stem/progenitor cell enrichment, leading to lung tumorigenesis [100]. Taken together these results clearly show that p27 has pleiotropic functions and therefore merely upregulating it without understanding cellular context may not be very effective.

In many cancers p27 levels are decreased as a result of deregulation of transcriptional, translational or post-translational pathways. Much effort has been expended into understanding these mechanisms with the end goal being to determine ways of upregulating nuclear p27 to prevent cell cycle progression. To briefly summarize these studies, which have been reviewed extensively elsewhere, p27 transcriptional regulation is complex, and involves both repression via oncogenic transcription factors such as c-Myc and Id3, as well as activation via FOXO transcription factors and E2F1 [101]. In addition to transcriptional regulation, p27 has been shown to be regulated via miRNAs such as miR-221/222, which are overexpressed in some tumors [102]. p27 is also extensively regulated at a post-translational level, including multiple phosphorylation sites that dictate localization, as well as that phosphorylation sites that stabilize the protein via inhibiting ubiquitination. Some of these most important sites include Ser10, Tyr74/88/89, Thr157, Thr187, and Thr198.

Beyond phosphorylation, p27 localization can also be regulated via protein-protein interactions, such as Jab1, which binds to p27 and induces its nuclear export and degradation in the cytoplasm [103]. Jab1 is an interesting potential target for a number of reasons. Jab1 expression in tumors is inversely correlated with p27 levels and overexpression is correlated with poor prognosis [104–107]. This proto-oncogene has also been linked to radiation resistance and cisplatin-resistance due to inhibiting several apoptotic and DNA repair pathways [108]. Apart from p27, Jab1 can induce degradation of other tumor suppressors such as Smad4 and p53 as well as inhibit DNA repair via HR pathways that involve Rad51 [109–111].

p27 proteolysis via the proteasome is regulated by Skp2 [112]. Skp2 is part of a larger SCF complex, comprised of cullin1, Skp1, ROC1/Rbx1 and requires an adaptor protein called Cks1. Together, this complex functions as an E3-ligase that regulates a number of substrates including p27, p21, p57, FOXO1 and c-Myc [113–116]. Towards the end of G1 phase, Skp2 recognizes p27 phosphorylated at Thr187, which serves as a degradation signal, allowing cells to enter S phase. However, in cancer cells, Skp2 can be amplified and/or overexpressed, leading to decreased p27 levels constitutively [117–119]. As a result of this reciprocal relationship, the concept of targeting Skp2 has been proposed, however these studies have not progressed well. Genetic studies using a Skp2 deficient mouse model have demonstrated that acute inactivation of Skp2 in the context of Pten or Arf heterozygosity induced

senescence but not in the Skp2-deficient mice without other oncogenic signals [120]. Senescence induction correlated with decreased tumorigenesis and p27 induction in the preneoplastic lesions that could be detected in some mice, providing additional rationale for the development of Skp2 inhibitors.

A high-throughput screen for inhibitors of Skp2 was performed using purified components of the complex and p27 as a substrate [121]. A compound designated CpdA was discovered to induce cell cycle arrest at low micromolar doses in multiple myeloma cells, and this led to caspase-independent cell death via autophagy. In addition, because low levels of p27 is associated with resistance to therapy in myeloma cells, CpdA was examined as a chemosensitizer, and was found to synergize with bortezomib, and overcome resistance to doxorubicin and melphalan. Despite this useful spectrum of activity however, further progress has not been made using this agent since it is difficult to make, and was not potent enough to use *in vivo* (required 5–10 μM dose in cell lines). More recently, another screen was performed using a chemical-genetics approach using automated microscopy to identify compounds that upregulate p27 in prostate cancer cell lines [122]. After several rounds of stringent validation, two compounds were identified – SMIP001 and SMIP004. These compounds are not broad proteasome inhibitors, but specifically target Skp2, resulting in elevated p27 and decreased CDK2 activity at low micromolar doses. At this point is it not known whether either of these compounds will be effective *in vivo*.

Targeting p27 presents a considerable challenge, despite the large body of knowledge regarding the mechanisms of its regulation and their redundancy. Some open questions include which of the upstream regulatory enzymes would be the best target to induce a sustained increase in p27 cells in tumor cells specifically. In some ways, the lack of complete specificity of substrates presents the largest conundrum. Assuming it would be possible to move one or more of the identified compounds (or a derivative) into the clinic, it is possible that some of the off-target effects may be undesirable e.g. in some contexts the upregulation of cyclin E or c-Myc may drive additional genomic instability. In fact there is data suggesting this might be the case using siRNA in A549 lung cancer cells targeting p27, Skp2 or the combination of both mRNAs [123]. In the dual-siRNA treated cells, there was an increase in centrosome number, abnormal mitoses/nuclear atypia, which could be attributed to an increase in both full length and low-molecular weight forms of cyclin E. In tumors, such as triple negative breast cancers, which already have LMW-E expression we propose that a Skp2-targeting strategy could be detrimental because of this off-target effect, unless combined with CDK inhibitors.

The other concern with Skp2 targeting relates to the specific genetic background of p27. As mentioned previously, p27 transcriptional silencing such as by miRNAs or promoter methylation are not uncommon events, and could co-exist with Skp2 overexpression. In this scenario even a potent Skp2 inhibitor would be ineffective at restoring p27 expression, with similar potential consequences as described above. In addition, because cyclin D-CDK complexes also bind p27, the level of p27 induction that may be necessary to slow down the cell cycle might be fairly high. Clearly, much work lies ahead in developing more potent inhibitors and understanding in greater depth the cellular contexts in which this strategy could be beneficial and which contexts to avoid.

14.3 Exploiting Normal and Cancer Cell Differences for Protection of Normal Tissue

One of the most challenging problems in cancer therapy involving cytotoxic chemotherapy is how to selectively kill tumor cells while sparing normal dividing cells. Previous work from our group has demonstrated two potential strategies that utilize cell cycle synchronization as a mechanism of selectively arresting normal cells. We proposed that using UCN-01, a staurosporine analog which was developed as a PKC inhibitor, but was later shown to inhibit other kinases including CDK1, CDK2 and CDK4 at low nanomolar concentrations could be used in this manner. UCN-01 has been found to induce a reversible G1 arrest in normal cells, while Rb-deficient tumor cells arrest in S phase instead [124]. Cytostatic doses of staurosporine (0.5–10nM) can also be used to arrest normal cells in G1 without any detectable effect on tumor cells [125]. Importantly, staurosporine priming does not compromise the ability for tumor cells to respond to cytotoxic therapies, while normal cells are arrested in G1 and therefore not responsive to chemotherapy that targets cycling cells.

More recently with the availability of specific CDK4/6 inhibitors that are more clinically relevant than staurosporine, this hypothesis has been revived and tested in mouse models using PD-0332991 [126]. Myelosuppression induced by platinum drugs and anthracyclines are one of the most life-threatening toxicities seen in cancer patients, due to both heightened risk of infection while immunosuppressed and also due to the subsequent chemotherapy delays or dose reduction, which can compromise treatment efficacy. A study was performed comparing two different mouse models of breast cancer, one with intact Rb signaling (the MMTV-neu model) and the other with inactive Rb (C3-Tag), to examine whether CDK4/6 inhibition could protect the hematopoietic progenitor cells from carboplatin-induced quiescence. The C3-Tag model which best resembles basal-like breast cancers, is Rb-deficient and, unsurprisingly, does not respond to CDK4/6 inhibition as a single agent. Carboplatin is highly active in basal-like breast cancer, though, and co-treatment with PD-0332991 did not protect tumor cells from death. However, these mice had reduced thrombocytopenia. In contrast, MMTV-neu mice, which have previously been shown to be dependent upon CDK4 and cyclin D, were sensitive to PD-0332991 as a single agent. In addition, when carboplatin was combined with PD-0332291, tumors grew back faster, indicating the CDK4/6 inhibition in this context protects tumor cells as well normal cells from toxicity, therefore not gaining any significant therapeutic index. These results suggest that CDK4/6 inhibitors may have a new utility – in tumors that are CDK4/6 sensitive, these drugs can be used for anti-tumor effect (and should be used separately from other cytotoxic therapies), and in other tumors that are insensitive (e.g. Rb deficient, p16 overexpressed), these drugs can be normal tissue protectors from other cytotoxic therapies. As a practical consideration as a result of these discoveries, we advocate for Rb mutation status to become one of the biomarkers tested routinely in the clinic.

14.4 Newer Cell Cycle-Related Targets for Therapy

In this last section, we briefly outline rationale for targeting other cell cycle-related proteins and review the state of drug development for each class of agent. Some of these proteins are intimately involved in mitosis regulation, and will be discussed separately.

14.4.1 PIM Family Kinases

One of the most interesting emerging targets in the PIM family of kinases, which regulate multiple pathways including the cell cycle. The PIM family of serine/threonine kinases consists of three isoforms that have a significant degree of sequence homology, but differ in their tissue distribution [127]. Overall these proteins are expressed throughout in hematopoietic progenitors as well as liver, spleen and other epithelial and mesenchymal tissues, and have considerable functional redundancy. PIM1 is the best studied member of this family, and is thought to be the most widely relevant gene (of the 3 PIM isoforms) in cancer.

The PIM1 gene was identified in the 1980s as a frequent proviral integration site for Moloney murine-leukemia virus (MuLV) which induced T-cell lymphomas in transgenic mice [128]. Subsequently PIM1 was shown to cooperate with c-Myc in inducing lymphomas in utero or around birth, whereas Eμ-Myc transgenic mouse crossed onto a PIM1 and PIM2-deficient background had delayed lymphomagenesis [129–131]. More recently, PIM family kinases have been discovered to be overexpressed or mutated in other solid tumors such as prostate, pancreatic, ER-negative breast cancer and head and neck squamous carcinomas as well as many leukemias and lymphomas (AML and CLL), leading to the question of whether they could be targeted [132–137].

PIM kinases are unusual in that they are constitutively active, but are regulated largely at the transcriptional and translational level [127–138]. A wide range of cytokines and growth factors can activate PIM kinases, mainly via the JAK-STAT pathway and NFκB pathways [139, 140]. Since the mRNA has a short half-life, inhibitors of JAK-STAT could potentially be used to inhibit PIM signaling as well. An emerging paradigm places PIM1 at the center of a cellular stress response, since PIM1 can be induced by hypoxia and DNA damage via various mechanisms. For example hypoxia can induce PIM1 expression rapidly in a HIF1α independent mechanism, as well as induce nuclear translocation [141, 142]. PIM1 induction in response to hypoxia promotes cell survival via inhibition of apoptosis and is linked to chemoresistance under these conditions. PIM1 can also be induced in response to DNA damage by Kruppel-like factor 5 [143]. In a study of head and neck squamous carcinoma patients, upregulation of PIM1 in response to irradiation was shown to be associated with a poor response [144]. Since EGFR expression is also correlated

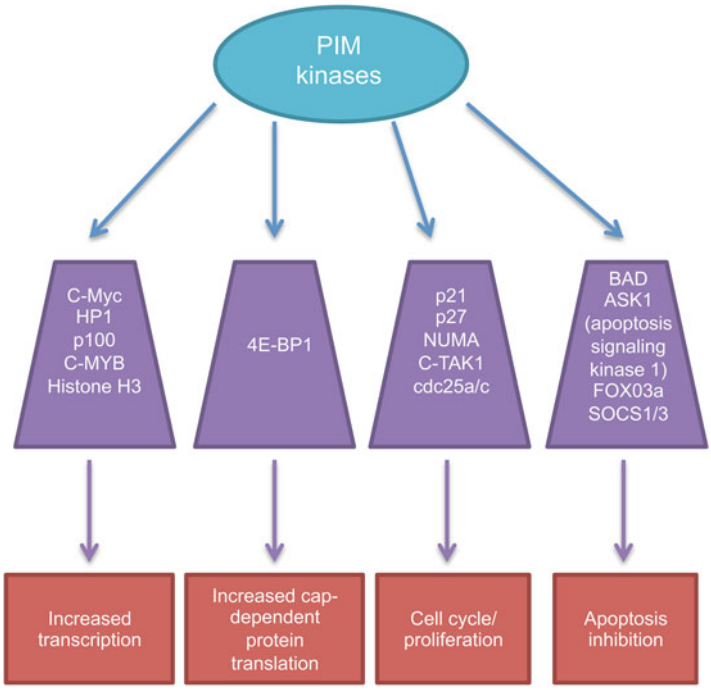

Fig. 14.4 PIM1 kinase substrates and cellular functions

with radiation resistance and EGFR is autophosphorylated and nuclear localized after IR, the authors asked with EGFR can regulate PIM1 levels/activity. In cell lines, EGF-ligands induced PIM1 nuclear-translocation, and this effect could be blocked by the EGFR antibody cetuximab or tyrosine kinase inhibitor, gefitinib. Similarly, HNSCC cells that were irradiated had more nuclear PIM1, and PIM1 knockdown demonstrated the pro-survival role that PIM1 plays in this context. Taken together, these studies show that PIM1 may be a good target in a number of different cancer systems.

PIM1 plays a number of cellular functions that all contribute to tumorigenesis, as depicted in Fig. 14.4. One of these is regulation of the cell cycle via phosphorylating several substrates such as p21, p27, cdc25A, cdc25C and HP1 [145–151]. One of the most robust readouts of PIM1 activity is phosphorylation of the pro-apoptotic protein BAD at Ser 112, which inactivates it, therefore enhancing anti-apoptotic activity of Bcl2 [152]. As mentioned previously, PIM1/PIM2 cooperates with c-Myc to regulate lymphomagenesis, and one of the mechanisms it does so is via phosphorylation of c-Myc which stabilizes the protein [153]. Overexpression of PIM1 also induces genomic instability via deregulating the mitotic spindle checkpoint, which causes abnormal mitoses, centrosome amplification and aneuploidy [154]. In hematopoietic malignancies in which PIM2 is highly expressed, 4EBP1 is also a target that is involved in promoting cap-dependent translation initiation of

Fig. 14.5 Structure of PIM1 kinase inhibitors

proteins that have growth promoting roles such as c-Myc and cyclin D1 [136]. In prostate cancer specifically, the androgen receptor is also a substrate of PIM1, and this phosphorylated form is transcriptionally inactivated and degraded [155, 156].

Structurally PIM kinases are distinct from other kinases in terms of how ATP binds to them, which has allowed chemists to design highly selective inhibitors. One of the most attractive features of PIM1 as a drug target is the lack of obvious phenotype in the knockout mouse, which is viable and fertile [157]. Compensation by other PIM family members is unlikely since compound knockout mice are also viable and fertile. The only phenotype that was observed in the $Pim1^{-/-}$ mouse is a subtle hematopoietic effect, such that the red blood cells are abnormally small but this did not lead to any physiological effects. When other potential hematopoietic functions were examined closely, it was found that bone-marrow-derived cells in culture had a significant impairment in IL-3 and IL-7 growth factor response [131].

The first compound that has been developed that has moved into cellular and *in vivo* studies is SGI-1776, which is an imidazo [1, 2-b] pyridazine compound that inhibits all three PIM kinases with IC50s of 7, 363 and 69nM (PIM1, PIM2 and PIM3 respectively), and has some activity against FLT3, another target in AML (see Fig. 14.5 for structure of this and other PIM inhibitors) [158]. In xenograft models of AML cells, this drug was highly active as an oral agent, inducing complete regression of blasts [159]. In addition, SGI-1776 can re-sensitize chemoresistant prostate cells to taxanes due to inhibiting multidrug resistance proteins including MDR1 [160]. Unfortunately when moved into phase 1 trials in humans (one trial was focused on prostate cancer and the other was non-Hodgkin's lymphoma) this drug was found to cause dose-limiting cardiac toxicity for reasons that are not clear, and the studies were stopped [161]. Another two structurally-related PIM1 inhibitors were identified in a chemical library screen called Smi-4a and Smi-16a which are benzylidene-thiazolidine-2, 4-diones [162]. When tested in vitro, these agents both had growth inhibitory activity in leukemia and prostate cancer cell lines, induced G1 arrest and induced p27 nuclear translocation. In addition, PIM1 inhibitors including Smi-4a synergize with both rapamycin in prostate cancer cells, and more recently with the Bcl2 inhibitor ABT-737 [163]. In our opinion despite these compounds having promising pre-clinical activity, the drug discovery market relating to PIM1 kinase is wide open right now. Special attention should be focused on the potential cardiac toxicity profile of future inhibitors (compared to SGI-1776), to try to understand what the off-target mechanisms are that underlie the Qt prolongation seen with SGI-1776 in patients.

14.4.2 Mitotic Kinases: Aurora Kinase Family

In much of this chapter so far we have discussed targets that function early in the cell cycle in regulating G1 and S phases. However, G2 and M phases are also very kinase-rich and have tremendous potential as drug targets. Several classes of chemotherapies already target these processes, such as taxanes, which bind to tubulin and disrupts the assembly of the spindle. In order to design better therapies against proteins that act in G2 and M phase, we must understand their functions at a mechanistic level and how they contribute to the events that are necessary for progression through these stages. During G2 phase when cells are preparing for mitosis, the cell is very active in ensuring the DNA was replicated correctly, and dividing the other organelles. In addition, microtubule proteins are being synthesized in order to form the mitotic spindle along which the chromosomes will segregate during mitosis. G2-M phase targets include proteins that are involved in entering mitosis (such as Aurora kinase A), the spindle assembly checkpoint (such as BUB1), and mitotic exit (such as APC). Each of these proteins and processes could be the focus of entire chapters, so we will provide a high-level overview of each here, and point the readers to recent reviews on these proteins.

Aurora kinases, of which there are three highly related isoforms (A, B and C) in mammalian cells, are key regulators of mitosis that have been well conserved throughout eukaryotic organisms. All three isoforms have been the focus of drug development over the past several years, and most of the inhibitors target two or three of them due to the highly conserved catalytic domain [164]. Aurora kinase A (AURKA) and Aurora kinase B (AURKB) have the strongest evidence for a role in tumor cell growth, whereas Aurora kinase C has scant evidence. This may be because for many years Aurora kinase C was thought to be primarily expressed in the testes where it plays a role in spermatogenesis, by playing similar roles to AURKB [165]. However more recently it has also been found to be expressed at high levels in some cancer lines, and several point mutations have been found in lung tumors but relatively little is known about its function [166, 167].

AURKA is ubiquitously expressed and is the first of the family members to be activated starting in late S phase and working through to completion of mitosis. Many related processes are regulated by AURKA, including maturation and separation of the centrosomes, assembling the mitotic spindle, chromosome alignment and cytokinesis [168–170]. Regulation of AURKA levels is also important for mitotic exit, as either too much or too little activity leads to failure of cytokinesis and multinucleation [171]. AURKA regulation occurs at both transcriptional and posttranslational levels, including activation by autophosphorylation at Thr288 on the activation loop, and deactivation via protein phosphatase 1 [172]. In cancer, AURKA is frequently amplified/overexpressed due to various mechanisms, especially in higher grade tumors and has been demonstrated to be a poor prognostic factor [173–176]. It was established as a bona fide oncogene when it was shown to be capable of inducing rodent fibroblast cell transformation due to formation of multipolar mitotic spindles that induce genomic instability [177]. Interestingly, these chromosomal

abnormalities that occur in AURKA overexpressing cells does not lead to cell death, because AURKA also promotes cell survival pathways including AKT-mTOR and nuclear accumulation of cyclin D1 [178]. NFκB is another anti-apoptotic pathway that is regulated by AURKA phosphorylation of its inhibitor, IκB [179]. AURKA has also been shown to interact with the p53 network, specifically via phosphorylating p53 inducing its degradation via MDM2, as well as phosphorylating p53 on Ser 215 which inhibits its DNA binding ability [180, 181]. These findings demonstrate the wide spectrum of roles that AURKA plays in cellular transformation and provide significant rationale for targeting this kinase.

Similar to Aurora kinase A, Aurora kinase B (AURKB) is also expressed in all proliferating cells, however it plays more limited roles as a chromosome passenger protein. AURKB is primarily expressed starting during prophase, where it is localized at the kinetochore to ensure correct chromosome alignment to the spindle and also helps ensure chromosomes segregate correctly [182]. In addition, AURKB phosphorylates Histone H3 at Ser10 and Ser28, which facilitates chromosome condensation [183, 184]. In cancer AURKB is not amplified, however it is still highly expressed in several tumor types [185–187]. Apart from regulating kinetochore-spindle interactions, in cancer cells, AURKB has been linked to degradation of p53 via phosphorylation at multiple sites [188], providing further rationale for targeting this kinase.

Inhibition of AURKA leads to G2 arrest, and has been shown to increase chemo- and radiosensitivity in cancer cells [189]. Quite a number of inhibitors have been generated by most of the major pharmaceutical companies and are currently being tested in early stage clinical trials. Many of these target both AURKA and AURKB, although Millennium has two AURKA specific compounds, MLN8054 and MLN8237. For a recent review with information about the clinical development of these agents, see [169]. Inhibition of AURKB are known as mitotic drivers, since they cause overriding of the mitotic checkpoints and results in aberrant mitosis and aneuploidy. This contrasts with AURKA inhibitors which block passage through mitosis. The question of whether inhibiting both AURKA and AURKB is better than either kinase alone has still not been answered. In preclinical genetic studies, the results have been equivocal. In one study in pancreatic cancer, antisense oligonucleotides to AURKA, AURKB or the combination were added to cells and responses compared [190]. The combination of both oligonucleotides was not better at inducing caspase activation, accumulating tetraploid cells or reducing formation in soft agar than either one alone. Targeting AURKA alone had a slightly better response overall versus AURKB alone, and this correlated with cells rounding up and detaching from the plate versus becoming large and multinucleated with the AURKB oligonucleotide. In contrast to this pancreatic study however, in colon cancer cells, AURKB inhibition was better than AURKA [191]. In order to move these targets forward, greater emphasis will have to be placed on understanding what contexts predict response to inhibition of each protein, and multiple readouts of each kinase inhibition should be analyzed since it is possible that each drug will have a slightly different profile. One clue that has already emerged is that p53-deficient cells more readily undergo apoptosis in response to the VX-680 inhibitor,

however since this is a pan-aurora inhibitor, it is difficult to dissect out which target is most relevant in p53-deficient tumors [192]. Further molecular and pharmacodynamic characterization of sensitive and resistant patients in the many clinical trials in progress should elucidate more such factors, as well as more detailed preclinical work with patient derived xenograft models should be the *in vivo* assay of choice in these studies.

14.4.3 Other Mitotic Targets of Interest

Moving forward as genomic studies are completed and more functional screens are performed it is likely more novel cell cycle targets will be found. Some examples of this nature that have been identified so far include MELK, Bub1, and Mps1. We will briefly summarize some of these proteins and how targeting them might be useful in cancer.

Maternal embryonic leucine zipper (MELK) is an atypical member of Snf1/AMPK family of kinases that has received only a little research attention so far. MELK is upregulated in several solid tumors including high-grade prostate cancer, astrocytoma, medulloblastoma and in breast cancer [193–195]. In addition, MELK is highly expressed in neural and breast cancer stem cells, making it a potentially attractive target to eradicate this population of cells thought to be the main drivers of drug resistance and eventual disease progression [196–198]. Expression of MELK is known to be increased in mitotically-arrested cells, and in prostate cancer cells is highly correlated with several other cell cycle/proliferation related genes including AURKB, cyclin B2 and DNA topoisomerase 2 alpha [193, 199, 200]. A few recent studies have suggested a role for MELK in radioresistance and chemoresistance, and have provided some in vitro evidence that knockdown can sensitize cancer cells to additional therapies [201, 202]. The only known pharmacological agent that targets MELK so far is the antibiotic siomycin A which reduces MELK expression and has been shown to decrease glioblastoma growth *in vivo* via targeting the neural stem cells [203].

The spindle assembly checkpoint (SAC) is a mechanism of delaying anaphase if the kinetochores are unattached to microtubules. There are at least 14 proteins involved in this process, four of which are kinases that are potentially targetable. These kinases are Bub1, BubR1, Mps1 and aurora B, although aurora B is dispensable for the checkpoint. If the SAC checkpoint is active, some of the components, such as Bub1, sequester Cdc20 which is the active part of the APC/C complex which degrades cyclin B. Bub1 may be a master regulator of the spindle assembly checkpoint by recruiting other important proteins involved such as BubR1, Mad1 and Mad2 [204]. The underlying concept behind targeting this checkpoint is that by preventing SAC activation, severe chromosome segregation occurs occur which causes cell death. Even partial inhibition of any of these essential mitotic checkpoint components can sensitize tumor cells to mitotic-targeting chemotherapies such as taxanes, whereas normal cells are not sensitized since normal cells can maintain a diploid population of cells [205]. Proof of this principle *in vitro* has been obtained for inhibitors of Mps1 [206, 207].

Cdc20 has also been proposed as a target in cancer due to the strong phenotypes seen in genetic studies from blocking mitotic exit. The cdc20 homozygous knockout mouse is embryonic lethal at the two-cell stage due to a metaphase arrest [208]. When an inducible knockout model was generated, a similar phenotype could be observed upon induction, and very high levels of cyclin B was observed in the cell, consistent with a defect in APC/C function [209]. When tumors of either epithelial or mesenchymal origin were induced in this model, and then cdc20 knockout was induced, the tumors rapidly regressed due to mitotic arrest and apoptosis.

In summary, there are various strategies that are being investigated to interfere with mitosis including delaying mitotic entry and spindle formation, preventing activation of the spindle assembly checkpoint or targeting mitotic exit via the APC/C-cdc20 complex. Such strategies may synergize with current chemotherapies that act in mitosis such as taxanes and vinca alkaloids, allowing lower doses of these agents to be administered. The question remains however whether a sufficient therapeutic index can be reached since normal cells also require these processes to be intact to undergo normal mitosis.

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Chapter 15
Histone Demethylases in Prostate Cancer

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Abstract Accumulating evidence has suggested that epigenetic alternations are as important as genetic mutations in cancer development. It is proposed that tumors are arisen by "malignant reprogramming" driven by a combination of both genetic and epigenetic changes. It therefore comes as no surprise that histone demethylases, the newest members of the histone modifying enzymes, are found to be targets of mutations and dysregulation in cancer cells. In this review article, we provide an overview of the types of histone demethylases whose genetic structure or expression is altered in cancers, the action of histone demethylases in cancer development and their potential inhibitors. Special emphasis is placed on the roles of histone demethylases in prostate cancer progression.

Keywords Histone demethylase • Inhibitor • Prostate cancer • Mutation • Epigenetics

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Abbreviations

AR	Androgen receptor
ARE	Androgen responsive elements
CDK	Cyclin-dependent kinase
CGH	Comparative genomic hybridization
CRPC	Castration-resistant prostate cancer
ER	Estrogen receptor
FAD	Flavin adenine dinucleotide
HIF	Hypoxia-induced transcription factor
HP1	Heterochromatin protein 1
KDM	Histone lysine demethylase
MEF	Mouse embryonic fibroblast
MAO	Monoamine oxidase
NOG	N-oxalylglycine
PRC	Polycomb repressive complex
SAHF	Senescence-associated heterochromatic foci

15.1 Histone Lysine Demethylase and Cancer

Chromatin is a highly ordered structure of eukaryotic DNA, which is packed into nucleosomes by core histone protein octamers: H2A, H2B, H3 and H4. Post-translational modifications of histone N-terminal basic tails cause conformational change of the nucleosome, allowing access of regulatory machineries to the DNA for transcription, replication, and repair [1, 2]. Several lysine residues on the histone tails can be mono-, di- or trimethylated. Depending on the position and degree of lysine methylation, the biological outcome is different. For example, histone marks such as H3K9me2, -me3 and H3K27me3, are involved in heterochromatin formation and gene silencing; while H3K4me3 is associated with actively transcribed genes. Because of the importance in gene expression, histone methylation and demethylation play critical roles in several biological processes, and altered histone methylation patterns are linked to human diseases such as neurological disorders and cancer.

Previously, histone methylation was believed to be irreversible. It was not until recently that the enzymes capable of removing methyl groups from histone were identified. As summarized in Table 15.1, histone lysine-specific demethylases (KDMs) have exquisite substrate specificities toward particular lysines on histones. KDMs can be broadly classified into two families: LSD1 (KDM1) and Jumonji C domain-containing (JmjC) family (KDM2 to KDM8). LSD1 family is amine oxidase which catalyzes demethylation through a flavin adenine dinucleotide (FAD)-dependent reaction. The JmjC family demethylase contains a conserved JmjC catalytic domain, which coordinates an electron shuffle between the methylated

Table 15.1 Histone demethylases with specific substrates identified

Name	Synonyms	Substrate
KDM1A	AOF2/BHC110/LSD1	H3K4me2/me1 H3K9me2/me1
KDM1B	AOF1/LSD2	H3K4me2/me1
KDM2A	JHDM1A/FBXL11	H3K36me2/me1
KDM2B	JHDM1B/FBXL10	H3K36me2/me1 H3K4me3
KDM3A	JHDM2A/JMJD1A/TSGA	H3K9me2/me1
KDM3B	JMJD1B	H3K9me2
KDM4A	JHDM3A/JMJD2A	H3K9me3/me2 H3K36me3/me2
KDM4B	JHDM3B/JMJD2B	H3K9me3/me2 H3K36me3/me2
KDM4C	JHDM3C/JMJD2C/GASC1	H3K9me3/me2 H3K36me3/me2
KDM4D	JHDM3D/JMJD2D	H3K9me3/me2
KDM4E	KDM4DL	H3K9me3/me2
KDM5A	JARID1A/RBP2	H3K4me3/me2
KDM5B	JARID1B/PLU1	H3K4me3/me2
KDM5C	JARID1C/SMCX	H3K4me3/me2
KDM5D	JARID1D/SMCY	H3K4me3/me2
KDM6A	UTX	H3K27me3/me2
KDM6B	JMJD3	H3K27me3/me2
KDM7	JHDM1D/KIAA1718	H3K9me2/me1 H3K27me2/me1
KDM8	JMJD5	H3K36me2
PHF2	JHDM1E	H3K9me2
PHF8	JHDM1F	H3K9me2/me1 H3K27me2 H4K20me1
JMJD6	PSR/PTDSR	H3R2 H4R3

lysine with the co-factors Fe(II), α-keto-glutarate, and molecular oxygen. This reaction ultimately results in the removal of the methyl group. Dysregulation of these enzymes alters the chromatin conformation and reprograms gene expression, which sometimes leads to malignant transformation of the cells. Table 15.2 presents a summary of recent literature on the topics of KDMs dysregulation in cancer. There are several other excellent reviews on this subject [3–5] which the readers may wish to refer to.

15.1.1 Dysregulation of Histone Demethylases in Cancer

Global alteration of histone methylation such as the loss of H3K4me2 and H4K20me3 are hallmarks of many cancers and are associated with poor prognosis [6–9]. Aside from the global changes, alterations of histone methylation patterns within specific cancer-causing loci also have consequences in cancer cell proliferation and survival. It is thus not surprising that mutations and aberrant expression of histone demethylases (Table 15.2) have been associated with carcinogenesis.

Table 15.2 Alterations of histone demethylases in cancer cell

Name	Alteration	Cancer associated	Target/Mechanism	References
KDM1A	Overexpression	Breast (ER-)	Regulates p21, ERBB2 and Cyclin A2 expression	[43]
	Overexpression	Non-small cell Lung	Regulates expression of EMT markers	[122]
	Overexpression	Prostate	AR coactivation	[98–101]
	Overexpression	Bladder		[123]
	Overexpression	Neuroblastoma	Maintains un-differentiated, malignant phenotype	[124]
	Overexpression	Nasopharyngeal carcinoma		[125]
	Underexpression	Hepatocellular carcinoma		[16]
	Underexpression	Breast	Inhibits invasion and metastasis through TGFβ signaling	[17]
KDM2A	Underexpression	Prostate	Represses transcription of centromeric satellite repeats. Sustain genomic stability	[13]
KDM2B	Overexpression	Leukemia	Silences p15(Ink4b)	[18]
	Underexpression	Brain, glioblastoma	Represses transcription of rRNA.	[14]
KDM3A	Overexpression	Prostate	AR coactivation	[103, 104]
	Overexpression	Bladder, Lung	Positively regulates G1/S transition via HoxA1 gene expression	[40]
	Overexpression	Hepatocellular carcinoma		[126]
	Overexpression	Kidney		[127]
	Overexpression	Renal	Regulates hypoxic gene expression	[68]
KDM3B	Overexpression	Leukemia	Induces leukemogenic oncogene expression	[128]
	Overexpression	Prostate		[104]
KDM4A	Overexpression	Breast	ER coactivation. Positively regulates Cyclin D1	[36]
	Overexpression	Prostate	AR coactivation	[12, 104, 106]
	Underexpression	Bladder		[129]
KDM4B	Overexpression	Colorectal cancer	Regulates hypoxia-inducible gene expression	[130]
	Overexpression	Gastric	Regulates p53 and p21	[52]
	Overexpression	Prostate		[12]
	Overexpression	Bladder, Lung	Promotes G1/S transition by positively regulating Cdk6	[37]
	Overexpression	Breast	ER coactivation. Positively regulates Cyclin D1 and MYC	[38]
	Overexpression	Renal	Regulates hypoxic gene expression	[68]

KDM4C	9p23-24 amplification	Breast	Regulates NOTCH1 expression	[23, 131]
	Overexpression	Prostate	AR coactivation	[12, 101]
	9p24 amplification	Lymphoma	Regulates MYC expression	[24]
	Overexpression	Oesophageal, Medulloblastoma		[22, 132]
KDM5A	Gene amplification	Breast (drug resistance)	Negatively regulates CDK inhibitors and apoptotic genes	[25]
	Overexpression	Gastric	Negatively regulates CDK inhibitors	[44]
	Translocation	Acute myeloid leukaemia	NUP98-PHD fusion. Forced transcription of developmental loci	[26, 27]
KDM5B	Overexpression	Prostate	AR coactivation	[104, 107]
	Overexpression	Breast		[133, 134]
	Overexpression	Bladder	E2F1 and E2F2 expression.	[49]
KDM5C	Inactivating mutation	Clear-cell renal cell carcinoma		[29]
	Overexpression	Prostate	Suppresses TGF-β/Smad3 signaling pathway	[113]
KDM6A	Inactivating mutation	Clear cell renal cell carcinoma		[29, 30]
	Inactivating mutation	Leukemia, Breast, Colorectal, Endometrium, Gliobastoma, Lymphoma, Myeloma, Lung, Esophageal, Pancreas		[30]
	Inactivating mutation	Bladder		[31]
KDM6B	Overexpression	Hodgkin's lymphoma		[135]
	Overexpression	Prostate		[136]
KDM8	Overexpression	Breast	Regulates cyclin A1 expression.	[42]
	Overexpression	Leukemia	Negatively regulates p53 expression.	[46]
PHF8	Overexpression	Prostate		[104]

15.1.1.1 Aberrant Expression

While some histone demethylases behave like oncogenes, others play tumor suppressive roles. KDM4 family members that are widely overexpressed in several tumor types are believed to be putative oncogenes because of their demethylation activity toward H3K9me3/me2, a histone mark crucial to maintaining the heterochromatin structure. The maintenance of heterochromatic structure not only is essential for gene expression regulation but also plays an important role for protection of chromosome integrity. Narita et al. reported that during senescence, the levels of H3K9me3/me2 are increased at senescence-associated heterochromatic foci (SAHFs), concomitant with the increased binding of Heterochromatin protein 1 (HP1) [10]. The authors showed that some of the E2F target promoters in fact, acquire heterochromatic features during senescence, resulting in a permanent shutdown of these genes. Therefore, dissociation of heterochromatin could result in re-expression of E2F target genes and an escape from cellular senescence. Peters' study on the other hand, revealed that decrease of H3K9me3 at pericentric chromatin results in loss of the heterochromatin structure, leading to severe chromosome mis-segregation and genomic instability [11]. Together, as is found in prostate cancer [12], dysregulation of KDM4 family therefore may function as oncogenes and contributes to tumorigenesis. Other oncogenic roles such as alteration of cellular signaling pathways, promotion of cell cycle, expression of oncogenes/repression of tumor suppressors, are often linked with overexpression of various demethylases in tumor (Table 15.2). In addition to KDM4, KDM1A, KDM3 and KDM5 families are found to be overexpressed in several types of tumor.

While fewer cases, down-regulation of demethylases with tumor suppressive roles in cancer has also been reported. KDM2A plays a crucial role in sustaining heterochromatin structure and genome stability by repressing transcription of the centromeric satellite repeats [13]. KDM2B on the other hand, transcriptionally represses ribosomal RNA genes whose expressions are in high demand for proliferating cancer cells [14, 15]. Underexpression of KDM2B results in increased cell sizes and proliferation in tumor, suggesting it being a tumor suppressor [14].

Making things more complicated were the observations that the roles of histone demethylases as oncogenes or tumor suppressors are cell context dependent. As described above, KDM1A is overexpressed in many cancer types (Table 15.2), and appears to play oncogenic roles. However, KDM1A is also found to be down-regulated in liver and breast cancer, where it inhibits tumor invasion and metastasis [16, 17]. Similarly, while KDM2B is down-regulated in brain and glioblastoma, and was proposed as a putative tumor suppressor [14], it is found to be highly expressed in various leukemias with an oncogenic function [18]. These findings suggest that the functions of histone demethylases in cancer are dictated by the loci they act upon and the cell-type specific cofactors they associated with. As such, the results have strong therapeutic implications and underscore the importance in understanding the target gene profiles and the associated mechanisms of histone demethylases in particular cancer types.

The mechanisms of dysregulation of histone demethylase expression are multitude including alterations at the level of transcription and post-transcriptional modifications or genomic alterations. Below we will discuss the genetic alterations of KDMs in cancers.

15.1.1.2 Gene Amplification

Comparative genomic hybridization (CGH) analysis revealed that the 9p23-24 region is frequently amplified in several tumors including esophageal cancer, breast cancer and lymphoma; while KDM4C (GASC1/JMJD2C) gene located at the 9p23-24 amplicon is overexpressed in these tumors [19–24]. Overexpression of KDM4C results in tumorigenic phenotypes, and is found to be associated with aggressive breast tumors [23]. Similarly, KDM5A gene located at 12p11 is found to be amplified in several tumors including breast cancer, and overexpression of which, contributes to cancer proliferation and drug resistance [25].

15.1.1.3 Gene Translocation

van Zutven et al. first reported chromosome rearrangements involving KDM5A (JARID1A) and NUP98 in acute leukemias [26]. This translocation results in a fusion product consisting of the N-terminus of NUP98 and the C-terminal PHD finger domain of KDM5A. In doing so, the PHD domain targets NUP98 to active chromatin region with H3K4me3 histone mark and prevents the recruitment of the repressive polycomb complex (PRC2) to the promoter, thus, enabling active transcription of the crucial developmental loci and eventually leading to the development of leukemia [27]. In this case, it is not the catalytic of demethylase, but rather the chromatin binding domain which participates in the oncogenic transformation.

15.1.1.4 Gene Mutation

Inactivating mutations in histone demethylases have been identified. H3K4me3 demethylase KDM5C (JARID1C), suggested to be a tumor suppressor [28], has several nonsense and missense mutations in clear cell renal cell carcinoma patients [29]. Similarly, systematic mutational screen of KDM6A (UTX) reveals that nonsense, frameshift, and deletion mutations are often identified in cancers including leukemia, lymphoma, myeloma, glioblastoma, breast, colorectal, endometrial, lung, esophageal, pancreatic, bladder and clear cell renal cell carcinoma [29–31]. These findings suggest that KDM6A is a tumor suppressor, consistent with its ability to demethylate H3K27me3, a histone mark whose elevation is often associated with malignancy, and to positively regulate Rb tumor suppressor and antagonize Notch signaling pathway [32–35].

15.1.2 The Functional Roles of Histone Lysine Demethylases in Cancer

Histone lysine demethylases affect a wide spectrum of cellular pathways. Based on the literature cited in Table 15.2, the following oncogenic pathways appear to be the most frequently dysregulated by histone demethylases.

15.1.2.1 Cell Cycle Regulation

One of the major oncogenic properties of histone lysine demethylases is their role in cell cycle regulation. Overexpression of several demethylases is linked to promoting G1-S progression and inducing tumor cell proliferation through positive regulation of S-phase cyclins and cyclin-dependent kinases (CDKs), and/or negatively regulating CDK inhibitors. Demethylases that are reported to induce expression of the S-phase cyclins, Cyclin D1, include KDM3A, KDM4A, KDM4B and KDM5B [36–40]; and those for Cyclin A1 expression includes KDM4B and KDM8 [41, 42]. Those which negatively regulate CDK inhibitors such as $p21^{Cip1/Waf1}$, are KDM1A, KDM5A, KDM5B and KDM8 [25, 43–46]; and $p27^{Kip1}$ by KDM5A and KDM5B [25, 47]. E2F transcription factors that play crucial roles in G1-S transition are also common targets of histone demethylases [43, 48, 49]. KDM1A positively regulates E2F1 gene expression, and also regulates its transcriptional activity by destabilizing the Rb regulator MYPT1 [43, 50]. PHF8 functions as a co-activator of E2F1 during G1-S transition by forming a complex with it, and upon recruitment to E2F1 target promoters, PHF8 removes the repressive H4K20me1 mark and consequently activates expression of the target genes [51].

Histone demethylase-mediated cell cycle control and proliferation is also found to channel through the p53-Rb axis [46, 48, 52–55] and senescence regulation (see below). In addition to transcriptional regulation, histone demethylase such as KDM4A directly regulates DNA replication by removing heterochromatin marks and increasing chromatin accessibility for replication machinery [56].

15.1.2.2 Senescence

Senescence is a process of irreversible cell growth arrest important for preventing excessive proliferation and functions as a suppression mechanism of tumorigenesis [57, 58]. *INK4b-ARF-INK4a* locus encodes $p15^{INK4B}$, $p16^{INK4A}$ and $p14^{ARF}$ proteins that sense stress signals and function as key regulators of senescence [59]. The *INK4b-ARF-INK4a* locus is normally silenced with H3K27 methylation by the polycomb complexes PRC1 and PRC2. When cells undergo senescence, the PRC complexes and H3K27me3 are lost from the locus, leading to expression of *INK4A*, *INK4B* and *ARF* [60]. Studies on KDM2B revealed a double regulatory mechanism for senescence and cell proliferation. KDM2B regulates senescence in part, by

directly binding to the *INK4b-ARF-INK4a* locus and demethylating the locus-associated H3K36me2 and H3K4me3 which results in the suppression of *INK4a* and *INK4b* [61, 62]. KDM2B also modulates the expression of H3K27 trimethyltransferase EZH2 by negatively regulating tumor suppressor miRNAs let-7b and miR-101. KDM2B-mediated up-regulation of EZH2 increases the suppressive histone mark of H3K27me3 on *INK4b-ARF-INK4a* locus and further contributes to silencing. When primary mouse embryonic fibroblasts (MEFs) undergo senescence, the KDM2B-let7-EZH2 pathway presents a feed-forward mechanism to ensure senescence: KDM2B is down-regulated, leading to expression of let-7b and miR-101 which in turn, represses EZH2 expression [63]. The ability of KDM2B overexpression in promoting immortalization and sustained proliferation of both *wild type* and *INK4a-ARF* null MEFs suggests its important roles in tumor initiation and development.

Rb and p53 tumor suppressors also play essential roles in senescence. They induce senescence by regulating the expression of CDK inhibitor p21, and formation of heterochromatin on E2F-responsive promoters [10, 64]. During senescence, global changes in histone modifications include increase of transcriptional silencing marks H3K9me3, H3K27me3 and H4K20me3; and loss of active histone mark H3K4me3/me2 [64]. As suggested above, overexpression of demethylases that are involved in the Rb-p53-p21 pathway, or in removing the transcriptional silencing marks globally or loci-specific, may contribute to the loss of the tumor-suppressing senescence mechanism.

15.1.2.3 Hypoxia

Hypoxia is a stage when a cellular demand of oxygen for metabolism exceeds the local blood supply. It occurs in tumor tissues where cell proliferation outgrows angiogenesis, resulting in local low concentration of oxygen in the high-cell dense regions of tumors. Tumor hypoxia has been reported to associate with poor prognosis and with resistance to radiotherapy and chemotherapy. Therefore understanding the survival mechanisms of tumor cells under hypoxic conditions is of significant importance in the design of therapeutic strategy. Hypoxia-induced transcription factor (HIF) complexes, consisting of α- (HIF-1α, HIF-2α or HIF-3α) and β- subunits, are the predominant hypoxia-responsive regulators that modulate adaptive gene expression aiming at restoring cellular oxygen homeostasis. Recent studies suggest cooperative actions between epigenetic regulators and HIF in response to hypoxia. These regulators include enzymes involved in DNA methylation, chromatin remodeling and histone modifications at the HIF promoter regions. Under hypoxic conditions, global levels of H3K4me3 and H3K9me2 have been demonstrated to be increased, whereas H3K27me3 level decreases [65, 66]. It is speculated that these chromatin modification signatures indicate flexible access of other chromatin modifiers and transcriptional regulators to promoters, in turn facilitating reprogramming of gene expression under transient hypoxia-reoxygenation conditions that often occur in tumor [65]. Several JmjC histone demethylases such as KDM3A, KDM4B,

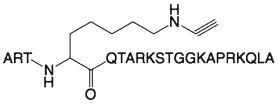
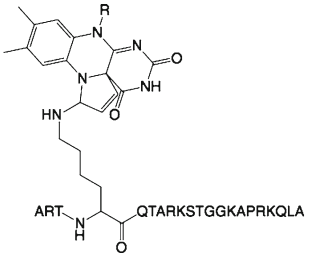
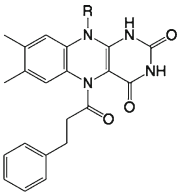
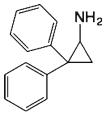
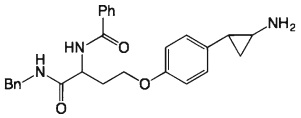
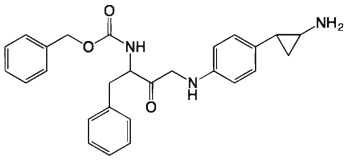
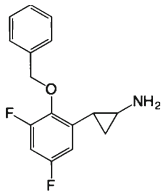
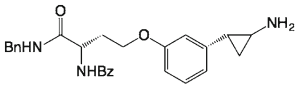
KDM4C, KDM5A and KDM5B are found to be activated in the HIF signaling pathway during hypoxic conditions [67–70]. The importance of hypoxia-induced histone demethylases is best illustrated by KDM3A (JMJD1A/JHDM2A). Upon induction under hypoxia, KDM3A is recruited to HIF target genes, and by removing the repressive H3K9me2 marks, KDM3A facilitates hypoxic gene expression such as adrenomedullin (ADM), differentiation factor 15 (GDF15), and GLUT3 (SLC2A3) [67, 71]. Mimura et al. further demonstrated that the recruitment of KDM3A to the GLUT3 promoter depends on physical interaction between KDM3A and HIF1 [71]. In addition to HIF, other transcription factors such as NFκB, CREB and EGR-1 are involved in hypoxia responses [72–74]. Given that many non-histone proteins including NFκB are found to be methylated, the roles of lysine demethylases in hypoxia may be broader than previously thought. Indeed, KDM2A is found to demethylate NFκB [75] and affects its transcriptional potential.

15.2 Inhibitors of Histone Demethylases

Given the strong implications of KDMs in cancer, small molecule inhibitors targeting their enzyme activities are being actively investigated in recent years (Table 15.3). Among them, inhibitors for KDM1A are the most extensively pursued; 12 such inhibitors (Compound 1 to 12) are listed in Table 15.3. KDM1A belongs to a superfamily of FAD-containing amine oxidases and the developed inhibitors include substrate analogues, MAO (monoamine oxidase) inhibitor analogues and polyamine analogues. Culhane et al. first reported compound 1 as a suicide inhibitor of KDM1A through substitution of lysine4 in H3 [76]. Through coupling of compound 1 with FAD, the mechanism of KDM1A demethylation was revealed ([77, 78], compound 2). The prototype of amino oxidases is the antidepressant MAO, based on which several inhibitors have been developed. Analogues of MAO inhibitors such as trans-2-phenylcyclopropylamine (compound 3-8) were shown to be effective in inhibiting KDM1A [79–85]. Another class of KDM1A inhibitors is polyamine analogues (compound 9-11) [86–88], inhibition of KDM1A in colon cancer cell by this class of compounds resulted in reexpression of aberrantly silenced genes [86, 87]. Compound 12, a new KDM1A inhibitor, was found to selectively target cancer cells with pluripotent stem cell properties [89]. Although these inhibitors are very useful in exploring the structure and biological function of KDM1A, their IC50s for cell killing are in the range of micromolar to millimolar, too high to be appropriate for clinical trials and hence further optimization is needed.

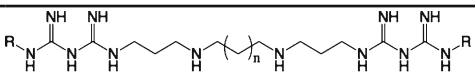
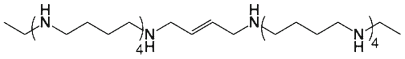
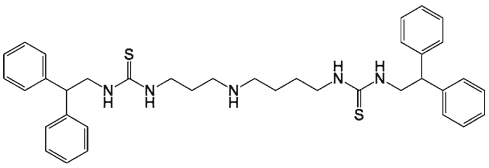
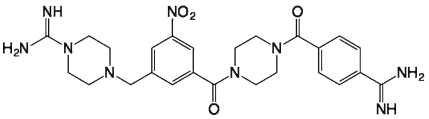
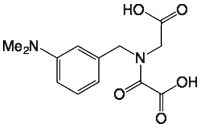
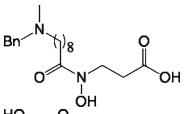
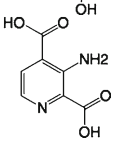
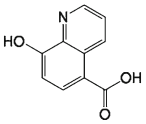
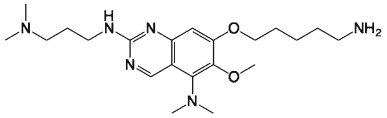
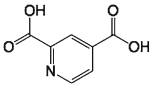
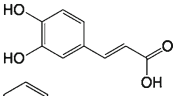
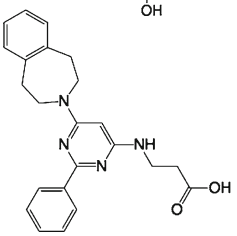
Other KDMs are JmjC containing demethylases, which are α-ketoglutarate dependent oxygenases. The design of most inhibitors is based on the scaffold of this cofactor. Compound 13, a NOG (N-oxalylglycine) analogue, was identified as a KDM4A inhibitor with IC50 of 3 mM. This compound is not cell permeable and methylation of the two hydroxyl groups is required for cell penetration [90]. Other KDM inhibitors include hydroxamate analogs, pyridine dicarboxylates and bipyridil compounds. Hamada et al. identified a series of hydroxamate analogues as KDM4A

Table 15.3 Histone demethylase inhibitors

Compound	Inhibitor structure	Targeted KDMs	IC_{50} (μM)	References
1		KDM1A	Suicide	[76]
2		KDM1A	Suicide	[77, 78]
3		KDM1A	2	[79, 80]
4		KDM1A	~250	[81]
5		KDM1A	1.9	[82]
6		KDM1A	1.3	[83]
7		KDM1A	1.0	[84]
8		KDM1A	1.6	[85]

(continued)

Table 15.3 (continued)

Compound	Inhibitor structure	Targeted KDMs	IC_{50} (μM)	References
9		KDM1A	2.5	[86]
10		KDM1A	~10	[87]
11		KDM1A	17.1	[88]
12		KDM1A	10.5	[89]
13		KDM4A	3,000	[90]
14		KDM4A KDM4C	2.0 1.3	[91]
15		KDM4E	0.11	[92]
16		KDM4A KDM4E	1.7 2.4	[93]
17		KDM7A	3.4	[94]
18		KDM5B KDM4A KDM4E	3 0.6 1.4	[93, 95]
19		KDM4C KDM6A	13.7 5.5	[96]
20		KDM6B	0.06	[97]

and KDM4C inhibitors in low micromole range ([91], compound 14), whereas 3-substituted pyridine 2,4-dicarboxylate was found to be a potent inhibitor of KDM4E ([92], compound 15). Through high-throughput screening, 8-hydroxyquinolines were identified as cell-active KDM4A and KDM4E inhibitors ([93], compound 16). Compound 17, a diazepin-quinazoline-amine derivative, selectively inhibits KDM7A ([94]). Interestingly, 2,4-pyridine-dicarboxylic acid (compound 18) was found to be an active inhibitor of KDM4A, KDM4E and KDM5A, indicating substrate similarities of these KDMs [93, 95]. Recently, catechols were reported to be active KDM4C and KDM6A inhibitors ([96], compound 19). The IC50s of most of these inhibitors are in millimolar or micromolar range, Kruidenier et al. identified compound 20 as a selective KDM6B inhibitor in nanomolar range. Interestingly, this inhibitor modulates the proinflammatory macrophage response ([97], compound 20). Although these inhibitors showed variable activities against purified KDMs, none of these inhibitors inhibit cancer cell growth below micromole range and none has reached clinical trials. Given the important function of KDMs in cancer, development of more potent KDM inhibitors is highly desirable.

15.3 Histone Lysine Demethylases in Prostate Cancer

Using the Oncomine database, we performed a comparison of several prostate cancer studies to identify histone demethylases that are differentially expressed in normal and tumor samples (Fig. 15.1). Our analysis agrees well with those reported in the literature (Table 15.2), and can serve as a future guide for developing demethylase-targeted therapies. The mechanisms regarding how the demethylases serve as progression factors in prostate cancer are being actively investigated, and below is a summary of the most relevant signaling pathways targeted by histone lysine demethylases.

15.3.1 Targeting Androgen Signaling

Androgen/androgen receptor (AR) signaling is essential for early stage of prostate cancer cell growth and survival, which can be managed by anti-androgen hormone therapy. At a later stage, the majority of the tumors are transitioned into hormone-independent or castration-resistant state (CRPC), for which there is thus far no effective cure. Although most of the CRPCs no longer depend on the external androgen for growth and survival, the AR activity which is often aberrantly activated is still required. Several histone demethylases overexpressed in prostate cancer (Table 15.2) contribute to the aberrant activation of AR and AR associated signaling. This is understandable, as nuclear hormone receptors such as AR are known to form complex with “co-activators” to exert their transcriptional function; these coactivators are often histone-modifying enzymes and chromatin remodeling

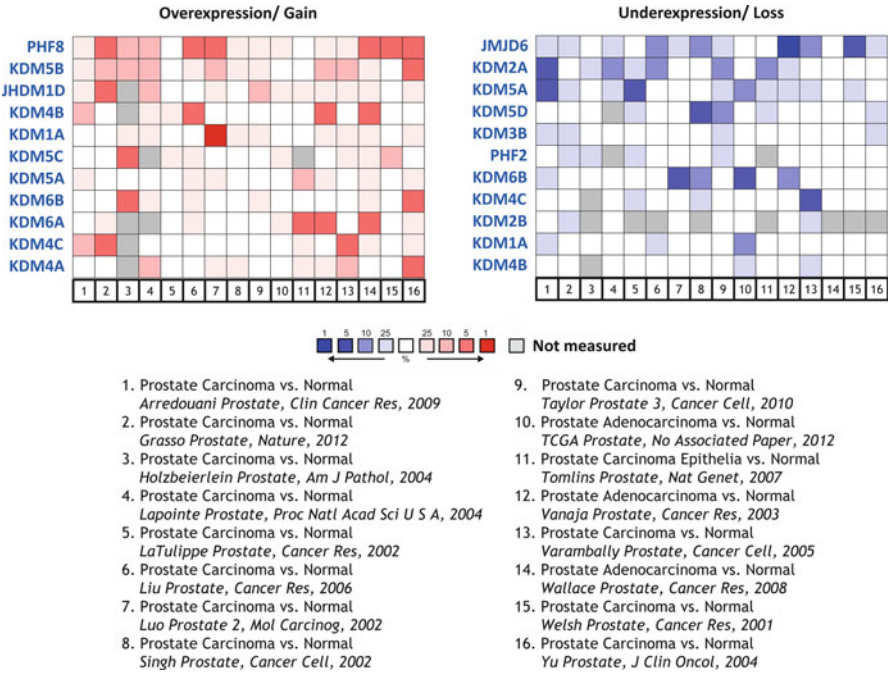


Fig. 15.1 Expression of histone demethylases that are differentially expressed in prostate cancer versus normal tissues are compared among 16 independent studies from Oncomine databases. In each dataset, the expression data was either based upon mRNA or DNA copy number analysis. *Left panel* shows the top 11 histone demethylase (half of total demethylases with substrates identified) that are gained/overexpressed in prostate tumors compared to that in normal tissue; *right panel* illustrates those that are loss/underexpressed in tumor. As described in Oncomine Differential Expression Analyses, each gene in each individual dataset is ranked to indicate its statistical significance of over- or under-expression. The ranking percentile of each demethylase relative to the ranks of all other genes in individual dataset is indicated with different color gradient: top 1 % of the ranks are shown with the darkest color (*red*, overexpression; *blue*, underexpression), the lower the ranking percentile, the lighter the color to be indicated. Briefly, the demethylases that are shown to be present with higher ranks (*darker color*) in more datasets suggests a higher probability of them being overexpressed or underexpressed in prostate tumors

proteins, to which histone demethylases belong. Their general functions are to generate an open chromatin conformation allowing RNA polymerase and transcriptional complex to engage and to transcribe the target gene. Aberrantly expressed histone methylases thus can cause aberrant activation of AR.

At least seven histone lysine demethylases are found to promote AR transcriptional activity: KDM1A, KDM3A, KDM4A, KDM4C, KDM4D, KDM5B and KDM8. KDM1A was originally described as a specific "eraser" of the active histone mark, H3K4me2/me1, and thus, functions as a transcriptional repressor. Interestingly, when it complexes with AR, and after H3T6 is phosphorylated by PKCβ1, KDM1A switches its demethylation specificity from H3K4me2/me1 to the

repressive histone mark H3K9me2/me1 [98, 99], thereby enhancing AR activity on the target genes. Inhibition or silencing of KDM1A results in reduced androgen-dependent proliferation and PSA (an AR target gene) transcription [98, 100]. These findings suggest that KDM1A functions as a coactivator for AR, and plays an important role in prostate cancer. Indeed, KDM1A is up-regulated in prostate tumor tissues, and overexpression of which is associated with higher relapse risk [100]. Wissmann et al. later identified a single complex consisting of KDM1A, KDM4C and AR, and reported KDM4C also as an AR coactivator. KDM4C and KDM1A bind to androgen responsive elements (ARE) located at promoter and enhancer of AR target genes, and upon hormone stimulation, they cooperatively remove the repressive tri-, di- and mono-methylated H3K9 marks [101]. This cooperation action synergistically enhances AR transcriptional activity on PSA enhancer. It is worth noting that different to the scenario of KDM1A-mediated estrogen receptor (ER) coactivation, where recruitment of KDM1A to ER target genes is ligand-dependent [102], chromatin binding of KDM1A and KDM4C to AR targets occurs in the absence of androgen treatment, while their demethylation activity on H3K9 depends on androgen signaling. KDM3A by contrast, displays hormone-dependent interaction with AR as well as chromatin recruitment to AREs [103]. Binding of KDM3A in turn, catalyzes loci specific demethylation of mono- and di-methylated H3K9. Similar to KDM1A, KDM3A and KDM4C are essential for hormone-induction of AR targets and hormone-dependent proliferation [101, 103]. Given their overexpession in prostate tumors and contributing to AR activation [12, 104], KDM3A and KDM4C are potential therapeutic targets for prostate cancer.

As exemplified by the analysis of AR and ER, recent studies have suggested that removal of the repressive H3K9 methylation marks at the promoter of target genes is crucial for nuclear receptor-mediated gene expression [105]. Aside from the KDM1A-KDM4C complex, H3K9me3/me2 demethylases KDM4A and KDM4D are also shown to interact with AR, and their overexpression enhance AR activity on the PSA enhancer [106]. Surprisingly, KDM5B (JARID1B) that demethylates the active histone mark H3K4me3/me2, is found to be overexpressed in prostate cancer and also serves as a coactivator for AR [104, 107]. While the detailed mechanism associated with KDM5B being an AR coactivator is not clear, at least two possibilities can be considered. First, as has been reported in different contexts [108, 109], H3K4me3/me2 may function as a repressive mark in a loci-specific way, and demethylation by KDM5B would activate AR target gene transcription. Second, similar to the case of KDM1A discussed above, association with AR could alter KDM5B's substrate specificity. The fact that KDM5B is significantly overexpressed in metastatic prostate cancer cells [107] indicates that the KDM5B-mediated AR activation is likely to bypass the androgen requirement, and KDM5B could serve as a potential target for late stage therapeutics. Our unpublished data showed that several other histone demethylases also interact with AR. We found, for instance, KDM8 (JMJD5), a H3K36me2 demethylase [42, 46], is overexpressed in high-grade prostate cancer and forms a complex with KDM4A and AR on chromatin. Ectopic expression of KDM8 and KDM4A synergistically enhanced AR activity with concomitant decrease of H3K36me2 at the target promoter. Because KDM4A

is also capable of catalyzing demethylation on H3K36me3/me2 [110, 111], the combination of KDM8 and KDM4A is expected to potently demethylate both H3K36me3/me2 and H3K9me3, allowing effective H3 acetylation. In addition to KDM8, we also identified interactions between KDM1B, KDM2A, KDM4B, KDM5A, KDM5D and AR. The data taken together suggest that histone demethylases either singly or in combination can serve as coactivator of AR to change the chromatin landscape of the AR target genes, thereby augmenting the transcription.

Finally, in addition to changing the local chromatin structure for AR target genes, histone demethylases are found to directly regulate AR. For instance, KDM1A is recruited by AR to an intronic enhancer of AR locus and represses AR expression via the removal of H3K4me3 mark [112]. Unlike the situation in PSA promoter, KDM1A in this case is not complexed with KDM4C and serves as a transcriptional repressor. This autoregulation takes place only when there is abundant androgen and acts as a feedback mechanism to shut off androgen signal. Under androgen-depleted conditions as in the case of CRPC, AR expression is usually increased. The discussion above indicates that histone demethylases, like histone acetylases and deacetylases, are partners of AR, and may play significant role in the dysregulation of AR activity during transition to hormone refractory prostate cancer. What discussed above is almost certainly only the tip of iceberg. More histone demethylases which directly or indirectly affect androgen receptor signaling are likely to be uncovered in the coming years.

15.3.2 Targeting Other Oncogenic Signals

KDM5C (SMCX/JARID1C) is a H3K4me3/me2 demethylase also found to be overexpressed in prostate cancer. KDM5C physically interacts with TGFβ-downstream transcription factor Smad3, and overexpression of which inhibits Smad3 activity independently of its demethylase activity [113]. Since TGFβ signaling acts as a tumor suppressive pathway in early prostate cancer [114], antagonizing the TGFβ-Smad3 pathway by KDM5C may therefore promote prostate tumor initiation. Another strongly overexpressed demethylase observed in clinical prostate cancer samples is PHF8, whose expression is correlated with high Gleason grade and poor prognosis [104]. PHF8 can demethylate multiple substrates including H3K9me2/me1, H3K27me2 and H4K20me1. Although the mechanism remains unclear, knockdown of PHF8 inhibits proliferation, migration and invasion ability of prostate cancer cells, indicating PHF8 as a potent oncogene for prostate cancer [104].

While a number of demethylases seem to exhibit oncogenic potential, KDM2A was found to be underexpressed in prostate cancer and functions as a tumor suppressor. Frescas et al. showed that KDM2A is required to maintain the centromeric heterochromatin state and also sustain genomic integrity. Underexpression of KDM2A in prostate cancer may thus cause genomic instability, contributing to cellular transformation [13].

15.3.3 Histone Methylation as Biomarkers for CRPC

Seligson et al. first reported that global levels of histone modification can be used to predict clinical outcome of prostate cancer patients with low Gleason grade [115]. Elevated H3K4me3 and H3K27me3, and reduced level of H3K9me2 in prostate tumor tissue are found to associate with poor prognosis [7, 116, 117]. While H3K4me1, H3K4me2 and H3K4me3 levels are significantly increased in CRPC, higher level of H3K4me1 is more likely to develop recurrence [118]. One of the mechanisms underlying the altered histone methylation-associated malignancy and prostate tumor recurrence is AR -mediated activation of proto-oncogenes and repression of tumor suppressors. Genome-wide analysis revealed that in CRPC cells, H3K4me1 and H3K4me2 are selectively enriched at enhancers of oncogenes such as *UBE2C* and *CDK1*, facilitating recruitment of AR for their transcription. Up-regulation of these cell cycle genes in turn, promotes growth of CRPC cells [119]. Similarly, increased H3K4me3 in prostate cancer cell correlates with the expression of oncogenes including *FGFR1*, *BCL2* and *HOXC5*[120]. By contrast, H3K27me3 mark is enriched at the promoters of tumor suppressor genes, leading to their silencing in metastatic prostate cancer cell [121]. Together, emerging studies have suggested that histone modifications can serve as prognostic markers to predict outcome of prostate cancer. The intervention potential of the possible demethylases and methyltransferases that are responsible for the altered histone methylation is worthy of further consideration.

15.4 Concluding Remarks

In the past 8 years since the discovery of the first histone demethylase, KDM1A, extraordinary progress has been made in understanding their modes of action on histones and their connections to epigenetic regulation of carcinogenesis. Epigenetic regulation of cancer is important not only during transformation and metastasis processes, but also during therapeutic resistance. As master programmers of epigenetic regulation, histone demethylases are potential targets for intervention. Attentions to this group of genes, especially on understanding of their up- and down-stream signal pathways will only increase in the coming years. A few comments on the future direction of these research activities are provided here. First, the early literatures on histone demethylases have mostly focused on their actions on histone. Yet, we now know that KDMs may have other cellular substrates whose demethylation fuel the carcinogenesis processes. Identification of non-histone substrates of KDMs will be important to fully appreciate KDMs' modes of action. Second, as exemplified by KDM5C, KDMs may exert their function in a demethylation-independent manner. Hence, small molecules targeting the enzymatic activity may not work in this case. Third, paradoxically, in some cancers, KDMs and their counteracting histone methylases can both be overexpressed and serve as progression factors (e.g., KDM8 and

NSD2). This suggests that it is not the global level of the particular histone marks, but rather the loci-specific epigenetic landscape which determines the final outcome. This makes the measurement of therapeutic responses more challenging. The development of histone demethylase inhibitors is still at very early stage; however several promising leads have already surfaced (e.g., KDM1A for prostate cancer). Given the wide range of activities and biological outcomes of histone demethylases, one can envision a tremendous surge of research activities in the related areas with an intensity which may rival those for tyrosine kinases.

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Chapter 16
Therapeutic Significance of Chromatin Remodeling Complexes in Cancer

Da-Qiang Li and Rakesh Kumar

Abstract The compaction of genomic DNA into chromatin has far-ranging consequences for almost all aspects of DNA metabolism activity. ATP-dependent chromatin remodeling complexes (CRCs) enable DNA-binding proteins access to nucelosomal DNA by altering chromatin structure through distinct mechanisms including nucleosome sliding, nucleosome assembly, and histone exchanges, in an energy-dependent manner. Consequently, CRCs play critical roles in diverse cellular processes that are dependent on chromatin template, including transcription, replication, and DNA repair. Thus, an aberration in these chromatin remodeling proteins leads to human diseases including cancer. In this chapter, we discuss the functional roles of CRCs in the regulation of gene transcription, DNA damage response, and its potential connection with cancer development as well as tumor therapeutics.

Keywords Chromatin remodeling • Transcription • DNA damage response • Cancer • Cancer development and progression • Cancer therapeutics

16.1 Introduction

It is increasingly accepted that cancer is a genetic disease. A precise understanding of how genetic alternations contribute to tumor development and progression is the key to develop effective strategies for winning the fight against cancer. The genetic

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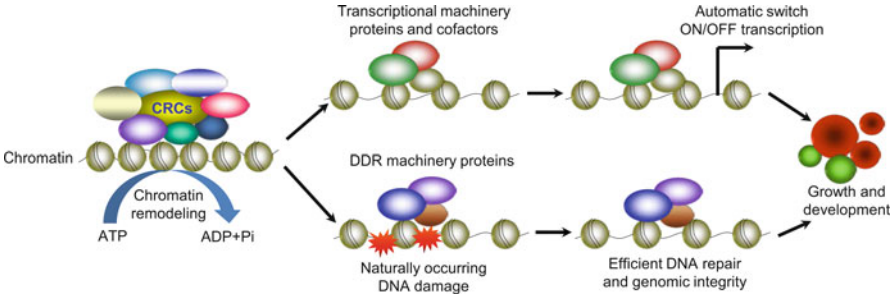

Fig. 16.1 Functional role of the CRCs in transcription and DDR. The CRCs alter chromatin structure in an energy-dependent manner. Consequently, the transcriptional and DDR machinery proteins get access to nucleosomal DNA and facilitate gene transcription and efficient DNA repair

material is stored into the nucleus in the form of chromatin. The fundamental building block of chromatin is the nucleosome core particle, which is made of approximately 147 base pairs of DNA wrapped around a histone octamer consisting of two copies of each of the four core histones H2A, H2B, H3, and H4 [1, 2]. The repeating nucleosome cores are connected by 20–80 base pairs of linker DNA and further assembled into hierarchically folded higher-order structures with the linker histone H1, nonhistone proteins and divalent metal ions [1–3]. By its very nature, the highly condensed structure of chromatin generally limits the accessibility of DNA binding proteins to the DNA, thus exerting an inhibitory effect on many critical DNA metabolizing activities, such as transcription, DNA replication, recombination and repair. To counteract this repressive barrier imposed by nucleosome architecture, eukaryotes have developed multiple intricate mechanisms to remodel nucleosomes, thus allowing DNA binding proteins such as transcription factors and DNA repair proteins access to the DNA. One of such mechanisms is involved in the ATP-dependent chromatin remodeling complexes (CRCs) that hydrolyze ATP to alter histone-DNA contacts through several mechanisms including nucleosome sliding, histone exchange, and nucleosome/histone eviction [4]. To date, four families of CRCs have been characterized in eukaryotes based on their compositions and functional domains, including the SWI/SNF (switching/sucrose non-fermenting), ISWI (imitation switch), Mi-2/NuRD (nucleosome remodeling and histone deacetylase), and INO80 (inositol requiring) [5, 6]. All CRCs are multisubunit complexes that contain an ATPase subunit [6] and most of them are conserved from yeast to humans. These CRCs play essential roles in many basic biological processes, including gene expression, DNA damage repair, and cell division (Fig. 16.1) [6–11]. Consequently, aberrations in these chromatin remodeling proteins are associated with a variety of human diseases including cancer. Thus, targeting the components of chromatin remodeling signaling pathways is currently being evaluated as a major therapeutic strategy in the prevention and treatment of human cancers. In the following sections, we focus on discussing the emerging role of CRCs in gene transcription, DNA damage response (DDR), and tumor development as well as its potential implication in cancer therapeutics.

16.2 Characterization of the CRC Family

The first discovered CRC family is the SWI/SNF, which was initially identified in independent screens for mutants affecting mating-type switching and growth on sucrose in 1994 [12–14]. The SWI/SNF family is composed of 8–14 subunits and is characterized by a bromodomain in its ATPase catalytic subunits, BRG1 (also known as SMARCA4 or BAF190A) and BRM (also known as SMARCA2 or BAF190B) http://www.genecards.org/ [15]. The bromodomain preferentially interacts with acetylated histones, which play both positive and negative roles in regulating the activity of the SWI/SNF remodeling factors [16]. In *S. cerevisiae*, this family contains the founding member SWI/SNF complex as well as the highly related RSC (remodel the structure of chromatin) complex [17]. Both SWI/SNF and RSC complexes exhibit a DNA-dependent ATPase activity to perturb nucleosome structure [17, 18], and contain nuclear actin-related proteins Arp7 and Arp9 [19, 20]. Arp7 and Arp9 form a stable heterodimer relying on their actin-related regions for heterodimerization, and function with DNA binding proteins to facilitate proper chromatin architecture and complex-complex interactions [19, 20]. Human complexes of this family have also been identified, including the BRG1-associated factor (BAF) complex and the polybromo BRG1-assocaited factor (PBAF) complex [21, 22]. With regard to homology, BAF1 is similar to the yeast SWI/SNF and PBAF is more like yeast RSC complex [21, 22].

The second family of CRCs is ISWI. One distinguishing feature of this family is that its ATPase subunit contains a carboxyl-terminal SANT and a SLIDE (SANT-like ISWI domain) domain, which together form a nucleosome recognition module that binds histone tails and linker DNA [23, 24]. The founding member of this family is the *Drosophila* NURF (nucleosome remodeling factor) complex, which was identified in 1995 by assaying the ability of drosophila embryo extracts to generate a nuclease-hypersensitive site within an array of nucleosomes [25]. NURF is composed of four distinct subunits, including the 140-kD ISWI ATPase subunit (NURF140) [26], a 55-kD WD repeat protein (NURF55) [27], the smallest NURF38 component [28], and the large NURF301 subunit [29]. In contrast to the SWI/SNF complex, the ATPase activity of NURF requires nucleosomes rather than free DNA or histones [25]. Thus, NURF acts directly on a nucleosome to alter chromatin structure by catalyzing nucleosome sliding, thereby exposing DNA sequences associated with nucleosomes [25, 30, 31]. Interestingly, the N-terminal histone tails are functionally important for modulating nucleosome mobility and regulating ATP-dependent nucleosome sliding by NURF [30]. In addition to NURF, the *drosophila* ISWI complex also contains the CHRAC (chromatin remodeling and assembly complex) [32] and ACF (ATP-utilizing chromatin remodeling and assembly factor) complexes [33]. Both exhibit chromatin assembly and nucleosome sliding activity in an ATP-dependent mechanism [32, 33]. In mammals, two highly related ATPase subunits of the ISWI CRC have been identified, including SNF2L (SNF2 like) and SNF2H (SNF2 homologue) [34]. Biochemical analysis revealed that the SNF2H ATPase catalytic subunit is contained in multiple complexes including ACF, CHRAC, RSF (remodeling and spacing factor), NoRC (nucleosome-remodeling complex), WICH (WSTF-ISWI chromatin remodeling), and WCRF (WSTF-related

chromatin-remodeling factor) [34–41]. In contrast, only a small number of complexes, such as the human NURF and CERF (CECR2-containing remodeling factor) complexes, contain the SNF2L ATPase subunit [35, 42].

The third family of CRCs is the Mi-2/NuRD complex, which was identified in1998 from several independent groups and processes both ATP-dependent chromatin remodeling and histone deacetylase activities [43–46]. The complex contains the histone deacetylases HDAC1/2, the histone-binding proteins RbAp46/48, the dermatomyositis-specific autoantigen Mi-2, the metastasis-associated proteins MTA1/2/3, and the methyl-CpG-binding domain proteins MBD2/3 [43, 44, 47]. Notably, Mi-2 subunit contains a chromodomain and plant homeo domain (PHD)-type zinc finger, and functions as a DNA-dependent, nucleosome-stimulated ATPase that remodels nucleosomes in an ATP-dependent manner [48]. In particular, Mi-2 lacking its chromodomains fails to bind or remodel nucleosomes [23].

The fourth family of CRCs is the evolutionarily conserved INO80 subfamily, which includes the INO80 complex and SWR1 complex [49]. The subfamily is characterized by a split ATPase domain and the presence of two RuvB-like proteins Rvb1 and Rvb2 [49]. The INO80 complex was initially purified from *S. cerevisiae* in 2000, consisting of about 12 subunits including Arp4, Arp5, Arp8, actin, and the Rvb1 and Rvb2 helicase proteins, and displays nucleosome-stimulated ATPase activity and ATP-dependent chromatin remodeling activities [50]. Deletion of Arp5 in yeast strains impairs INO80 ATPase activity, DNA binding, and nucleosome mobilization [51]. Similarly, Arp8 forms a complex with nucleosomes via the H3 and H4 histones [51, 52] and is essential for activity of INO80. In this context, deletion of Arp8 results in loss of INO80 function with multiple effects on cellular processes such as double-strand break (DSB) repair and chromosome alignment [52–55]. In contrast, Rvbp1p/Rvb2p is required for the complete assembly of a functional INO80 complex and for recruiting Arp5p to the INO80 complex in an ATP dependent manner [56]. The highly-related SWR1 complex was identified from *S. cerevisiae* in 2004 [57] and its human counterparts, termed Snf2-related CREBBP activator protein (SRCAP) and p400, were also identified afterward [58, 59]. In *S. cerevisiae*, this SWR1 complex contains Swr1p, a putative Swi2/Snf2-related ATPase, and 12 additional subunits. Among them, several subunits including Act1, Arp4, Rvb1 and Rvb2 are common to the INO80 complex [50]. Despite highly related to INO80, the SWR1 complex is unique in its ability to catalyze the incorporation of the histone variant H2AZ (Htz1 in *S. cerevisiae*) into nucleosomes [57, 60, 61], and this occurs in vitro in a stepwise and unidirectional fashion and requires dual activation with histone H2AZ and canonical nucleosome [62].

16.3 CRCs in Gene Transcription

The compaction of DNA into chromatin in the eukarytotic nucleus poses many obstacles to transcription [63]. The CRCs bind directly to nucleosomes and disrupt histone-DNA interactions using the energy of ATP hydrolysis, thus facilitating the access of the core transcription machinery proteins and general cofactors to nucleosomal DNA.

As a result, CRCs play a fundamental role in modulating transcription in yeast and higher eukaryotes. Notably, these CRCs have a range of specific and context-dependent roles in control of gene expression depending on the circumstance.

16.3.1 The SWI/SNF Complex

The SWI/SNF complex is involved in a variety of functionally distinct complexes and exerts diverse roles in gene regulation and genome function [64]. One outstanding example is that the SWI/SNF CRC participates in promoting transcriptional activation by nuclear receptors. The androgen receptor (AR) is a ligand-dependent transcription factor whose activity is tightly regulated by interacting cofactors and cofactor complexes and is a key player in prostate cancer development and progression [65, 66]. Considerable evidence has pointed out that the SWI/SNF CRC directs AR-mediated transcriptional activation, and different AR targets show disparity in the requirement for SWI/SNF [65–67]. A case in point is the BAF57 (also known as SMARCE1) subunit, which directly binds to the AR and is recruited to endogenous AR targets upon ligand activation, thus regulating AR activity, coactivator function, and AR-dependent proliferation [68]. Similarly, the BAF57 subunit specifically regulates estrogen receptor alpha (ERα)-dependent gene expression and proliferation in human breast cancer cells [69, 70]. Consequently, mutations in BAF57 deregulate several oncogenic signaling pathways, thus contributing to the development of breast cancer [71, 72].

In addition to BAF57, the BRG1subunit is a critical modulator of transcriptional regulation in various tissues and pathophysiological conditions [73]. For instance, BRG1predominantly interacts with Smad2 and Smad3 and is specifically required for transforming growth factor β-induced expression of endogenous Smad2/3 target genes through recruitment to Smad-dependent promoters [74, 75]. BRG1, as well as BRM, associates with the *CD44* and *E-cadherin* promoters and promotes their transcriptional activation in cancer cells through deceasing DNA methylation at their promoters [76]. In addition, SNF5 (also known as Ini1, BAF47, SNR1, or SMARCB1) mediates BRG1 recruitment to the $p15^{INK4b}$ and $p16^{INK4a}$ promoters and activates their expression through eviction of polycomb group silencing complex and extensive chromatin reprogramming [77].

Although the SWI/SNF CRC is generally associated with transcriptional activation, emerging evidence points out its additional role in transcriptional silencing pathway [78]. For instance, SWI3B, an essential subunit of the SWI/SNF complex, physically interacts with a long noncoding RNA (lncRNA)-binding protein, IDN2, and contributes to lncRNA-mediated transcriptional silencing [78]. Human BRM is functionally linked with the methyl-CpG binding protein MeCP2-depenendent transcriptional silencing [79]. Both BRG-1 and SNF5 subunits repress transcription of *cyclin D1* gene through the direct recruitment of histone deacetylase (HDAC) activity to its promoter, thereby exerting their tumor suppressor functions [80, 81]. More interestingly, BRG1 and BRM can switch their mode of function at same promoter between activation and repression through the context-dependent reprogramming of the SWI/SNF complex [82].

16.3.2 The ISWI Complex

The ISWI complex can space nucleosomes, thus affecting a variety of nuclear processes including transcription. Genome-wide analysis demonstrates that ISWI binds both genic and intergenic regions, and remarkably, binds genes near their promoters causing specific alterations in nucleosome positioning at the level of the transcription start sites [83]. Accumulating evidence suggests that the ISWI containing NURF complex is able to facilitate transcriptional activation via remodeling of chromatin in vitro and in vivo [29, 84, 85]. However, NURF also functions as a corepressor of a large set of JAK/STAT target genes in *drosophila* to regulate innate immunity network [86]. Similarly, ISWI and ACF1 directly repress Wingless transcriptional targets in *drosophila* [87]. In *S. cerevisiae*, Isw1 also functions in stress-induced gene repression under normal growth conditions [88]. In contrast, the Isw2 complex represses transcription of early meiotic genes during mitotic growth and this repressor function of lsw2 complex is largely dependent upon Ume6p, which recruits the complex to target genes [89, 90]. Subsequent studies further demonstrate that Isw2 acts as a transcriptional repressor by altering nucleosome positions, and loss of Isw2 activity results in the generation of both coding and noncoding transcripts due to inappropriate transcription [91].

16.3.3 The Mi-2/NuRD Complex

Accumulating evidence has uncovered a number of interesting connections between the Mi-2/NuRD complex and gene regulation [9, 92, 93]. A case in point is the metastasis-associated protein 1 (MTA1), the founding member of the MTA family, which was isolated by differential cDNA library screening using a rat mammary adenocarcinoma metastatic system [94]. MTA1 functions not only as a transcriptional repressor of estrogen receptor α [95], p21WAF1 [96], breast cancer type 1 susceptibility protein [97], RING finger protein 144A [98], phosphatase and tensin homolog [99], transforming growth factor β signaling component SMAD7 [100], guanine nucleotide-binding protein G(i) subunit alpha-2 [101], and homeobox protein SIX3 [102], but also as a transcriptional activator for certain genes, such as the breast cancer-amplified sequence 3 [103] paired box 5 [104], tumor suppressor alternative reading frame [105], cell surface oncogenic protein hyaluronan-mediated motility receptor [106], proto-oncogene protein Wnt-1 [107], and tyrosine hydroxylase [108]. One unanswered question in this field is what is the underlying mechanism for the physiologic switch between coactivator and corepressor functions of MTA1. It is becoming increasingly clear that post-translational modifications might play a role in the regulation of MTA1 function in transcription. In this context, SUMOylation and SUMO-interacting motif of MTA1 synergistically regulate its co-repressor activity on *PS2* transcription [109]. Similarly, acetylation status of MTA1 might also be crucial for its corepressor function on a negative modifier of

Ras activation and its oncogenic activity [101]. More interestingly, methylation of lysine 532 in MTA1 protein seemly represents a molecular switch between coactivator and corepressor [110]. In this context, methylated MTA1 is required for the NuRD repressor complex, while demethylated MTA1 recognizes the active histone mark and recruits coactivator complex onto its target gene promoters in a signaling-dependent manner [110].

16.3.4 The INO80 Complex

Involvement of the INO80 complex in transcription was first discovered in *S. cerevisiae*, in which INO80 facilitates transcription in vitro and in vivo [50, 111]. Subsequent studies further demonstrate that its mammalian orthologue also promotes transcription with transcription factor Yin-Yang-1 (YY1) [112]. In contrast, TBP-interacting protein 49b (TIP49b), a component of the INO80 complex, inhibits transcription factor 2 (ATF2) transcriptional activities in response to stress and DNA damage [113].

16.4 CCRs in the DDR

In response to DNA damage, chromatin undergoes a marked reorganization in an energy dependent manner, thus facilitating the DDR machinery proteins to recognize and repair the damaged DNA [114]. In addition to their putative roles in transcription, CCRs are intimately linked with the DDR.

16.4.1 The SWI/SNF Complex

The SWI/SNF complex is required for DNA replication [115, 116], somatic recombination [117], nucleotide excision repair (NER) [118, 119], and DSB repair [120]. SWI/SNF also regulates checkpoint activation after ultraviolet (UV) damage via regulation of the proliferating cell nuclear antigen-binding proteins Gadd45a and p21 [121]. The highly related RSC complex is also linked with efficient DSB repair [122, 123]. Interestingly, two isoforms of this complex, defined by the presence of either Rsc1 or Rsc2, play distinct roles in DDR and that at least part of the functional specificity is dictated by the bromo-adjacent homology (BAH) domains [124]. Moreover, the RSC and SWI/SNF chromatin remodelers play distinct roles in DSB repair; SWI/SNF is required during the early steps of homologous recombination (HR), while RSC is important upon the completion of the repair process [125].

16.4.2 The ISWI Complex

SNF2H, the catalytic subunit of ISWI complex, is rapidly recruited to DSBs in a poly(ADP-ribose) polymerase 1 (PARP1)-dependent manner and facilitates the RNF168-dependent signaling and repair of DSBs [126]. Similarly, the ACF1 chromatin remodeling factor accumulates at UV-induced DNA damage sites immediately following UV radiation [127] and promotes NER of UV-induced DNA lesions [128]. Similarly, the ACF1 complex accumulates rapidly at DSBs and is also required for non-homologous end joining (NHEJ) repair of DSBs in human cells [129]. Rsf-1 (also known as HBXAP) protein interacts with SNF2H to form an ISWI complex, RSF, and has been reported as an amplified gene in human cancers, including the highly aggressive ovarian serous carcinoma [130]. Emerging evidence shows that Rsf-1induces DNA damage and promotes genomic instability [131], and consequently, high-grade ovarian serous carcinomas, especially those with Rsf-1 overexpression, exhibit high levels of the DDR [132]. These findings highlight that increased Rsf-1 expression in tumors can induce chromosomal instability probably through DDR [131].

16.4.3 The Mi2/NuRD Complex

The initial link between the NuRD complex and DDR was found in 1999 [133], when Schmidt and colleagues discovered that ataxia telangiectasia and Rad3-related protein (ATR), a master regulator of the DDR, associates with multiple components of the NuRD complex, including MTA1, MTA2, HDAC1, HDAC2, and CHD4 [133]. Afterward, van Haaften G et al. in 2006 defined a role for *C. elegans* early growth response protein 1 (Egr-1), the homologue of human *MTA2* gene, in cellular sensitivity to ionizing radiation (IR) using a genome-wide RNA interference screening [134]. In 2009, Li et al. further discovered a previously unknown role for MTA1in IR-induced DSB repair and cell survival using MTA1-knockout fibroblasts [135]. In 2010, several studies from four different groups simultaneously reported a conserved role of the NuRD complex, including MTA1, MTA2, CHD4, HDAC1, and HDAC2 in DDR and DNA repair in multiple model systems [136–140].

The PARP family of proteins has been implicated in recruitment of proteins to sites of damage and is known to localize rapidly to sites of damage [136, 141]. In support of our early findings, emerging evidence shows that MTA1 is recruited to sites of DNA damage in a PARP-dependent manner, and depletion of MTA1 by siRNAs renders cells sensitive to IR, further highlighting its importance in promoting DNA repair [136]. The human homologue of *egr-1*, MTA2, also protects human cells against IR, suggesting its conserved role in the DDR [139]. CHD4 is rapidly recruited to DSBs in a PARP-dependent manner [136, 138], where it promotes RNF8/RNF168-mediated histone ubiquitylation and the ubiquitin-dependent accumulation of RNF168 and BRCA1 at sites of DNA lesions [137, 139]. CHD4 also

acts as an important regulator of the G1/S cell-cycle transition by controlling p53 deacetylation [138]. Consequently, loss of CHD4 causes defects in DNA repair and checkpoint activation, resulting in accumulation of spontaneous DNA damage and increased IR sensitivity [138, 139]. Furthermore, human HDAC1 and HDAC2 also function in the DDR to promote NHEJ repair [140]. Consistently, HDAC inhibitors block the activity of HDAC1 and HDAC2, resulting in defects in the DDR and hypersensitivity to the DSB-inducing agents [140]. Taken together, the NuRD chromatin–remodeling complex is a novel DDR factor that helps to preserve genome stability by regulating signaling and repair of DNA damage [11, 142]. Interestingly, recent studies pointed out that multiple NuRD components are lost during premature and normal ageing, resulting in accumulation of DNA damage during ageing [143], which could contribute to aging-related genomic instability and cancer [144].

16.4.4 The INO80 Complex

In addition to their well-established role in regulating transcriptional processes, accumulating evidence shows that INO80 and SWR1 chromatin remodeling components are essential for maintaining genomic integrity [10]. The INO80 complex is recruited to sites of DSBs through a specific interaction with the DNA damage-induced phosphorylated histone H2A (termed γH2AX) [145, 146], and mediates DSB repair through its role in DNA end strand resection [147]. INO80 is also recruited to sites of UV lesion repair through interactions with the NER apparatus and promotes the removal of UV lesions by the NER pathway [148, 149]. Moreover, INO80 is required for the restoration of chromatin structure after repair in response to UV-induced damage [149]. Interestingly, INO80 also shapes the DNA replication landscape. In this context, INO80 complexes are enriched at sites of replication and are required for efficient replication of late-replicating regions during replication stress through regulating S-phase checkpoint activity [4, 150]. INO80 also regulates the threshold of DNA damage during replication phase via modifying PCNA ubiquitination and Rad51-mediated processing of recombination intermediates at impeded replication forks [151–153].

16.5 CRCs in Cancer Development and Progression

16.5.1 The SWI/SNF Complex

Given its central function in epigenetic chromatin remodeling mechanisms, it is not surprising that alternation of the SWI/SNF CRC plays an important role in tumor development and progression. A substantial body of evidence indicates that several components of the SWI/SNF complexes function as tumor suppressors or negative

regulator of cellular proliferation [21, 154, 155]. One such example is the SNF5 core subunit, which has been documented to be mutated or inactivated in a number of human cancers including rhabdoid, rhabdomyosarcoma, epithelioid sarcoma, chronic myeloid leukemia, medulloblastomas, choroid plexus carcinomas, and melanoma [156–161]. In support of this notion, haploinsufficiency of SNF5 predisposes to malignant rhabdoid tumors in mice, and loss of SNF5 results in highly penetrant cancer predisposition with 100 % of mice developing T cell lymphoma or rhabdoid tumors with a median onset of only 11 weeks [162]. Collective evidence establishes that the tumor suppressor activity of SNF5 depends on its regulation of cell cycle progression, cell survival and senescence [163–168]. Inactivation of the SNF5 tumor suppressor stimulates cell cycle progression and cooperates with p53 loss to accelerate oncogenic transformation and tumor growth in mice [169, 170]. The inhibition of RhoA-dependent migration is another crucial tumor suppressor function of hSNF5, and its loss-of-function may lead to increased invasiveness and metastatic potential of cancer cells [171].

Another example is the ARID1A (also known as BAF250A, SMARCF1, p270, or hOSA1), which encodes a human homolog of yeast SWI1. The significance of ARID1A loss or mutation in cancer is now subject to intensive investigation. In this context, mutation of the *ARID1A* gene has been widely described in a broad array of tumor types, including gynecologic ovarian and endotrial carcinomas, pediatric Burkitt lymphoma, gastric carcinoma, breast cancer, and hepatitis B virus-associated hepatocellular carcinoma [172–178]. Consistently, restoring wild-type ARID1A expression in cancer cells that harbor ARID1A mutations is sufficient to suppress cell proliferation and tumor growth in mice [172, 175]. In contrast, ARID1A knockdown significantly promotes the proliferation, migration and invasion of cancer cells [173]. Functional evidence further points out that ARID1A collaborates with p53 to regulate p21WAF1 and SMAD family member 3 [179]. Together, accumulating genomic and functional evidence strongly supports classification of ARID1A as a tumor suppressor [177]. Similar to ARID1A, ARID1B (also known as BAF250B, or hOsa2) also inhibits cell growth and regulates cell cycle arrest through differentially regulating *c-myc* and *p21WAF1* gene expression [180].

In addition, loss or inactivation of BRG1, BRM, BAF155/SMARCC1, BAF180, and BAF200/ARID2 expression represents another mechanism for SWI/SNF complex in the development in human cancers, including hepatitis C infection-related liver cancer, melanoma, lung, pancreatic, skin, and breast cancers [21, 181–188]. Notably, BRG1 and BRM are silenced by different mechanisms. BRG1 is commonly silenced by loss-of-function mutations, whereas epigenetic silencing is a major mechanism for the loss of BRM in human cancer cells [188].

16.5.2 The ISWI Complex

A well-studied example is the Rsf-1, which plays an important role in cellular growth, survival, and oncogenic transformation, and its up-regulation is closely

associated with disease aggressiveness and poor prognosis in patients with various types of human cancers including bladder, colon, nasopharyngeal, gallbladder, oral, and ovarian carcinomas [130, 189–197]. A mechanistic study demonstrates that Rsf-1 interacts and collaborates with cyclin E1 in neoplastic transformation and p53 mutations are a prerequisite for tumour-promoting functions of the RSF/cyclin E1 complex [194]. In contrast, overexpression of Rsf-1 is rare in breast cancer, indicating that Rsf-1 is not a critical gene in breast cancer development [130, 198]. In contrast, SNF2L, a mammalian ISWI ortholog, suppresses cell proliferation and migration in human HeLa cells by attenuating Wnt signaling [199].

16.5.3 The Mi2/NuRD Complex

Of all the NuRD complex subunits, the MTA family members are best studied in the context of cancer development [92, 93]. MTA1, the founding member of the MTA1 family, has been documented to be overexpressed in a variety of human cancers and is significantly associated with tumor progression and poor clinical outcome [92, 93]. In contrast, the information concerning the expression of MTA2 and MTA3 in human cancers is limited. Like MTA1, increased expression of HDAC1 and HDAC2 has been documented in a variety of human cancers and linked with therapeutic resistance [200–202]. In contrast, lysine-specific demethylase 1, a newly identified component of the Mi-2/NuRD complex, inhibits the invasion of breast cancer cells in vitro and suppresses breast cancer metastatic potential in vivo [203].

16.5.4 The INO80 Complex

Although the function of the INO80 complex in transcription and DDR, its connection with human cancers is rarely reported. The SRCAP, a homolog of Swr1 in human cells, modulates expression of prostate specific antigen and cellular proliferation in prostate cancer cells [204]. Similarly, p400, another Swr1 homolog, inhibits p53-mediated *p21WAF1* transcription and the development of premature senescence [205]. P400 is an essential E1A transformation target that plays a major role in the E1A transforming process [206].

16.6 CRCs in Cancer Therapeutics

Glucocorticoids are used in the curative treatment of acute lymphoblastic leukemia (ALL) and resistance to glucocorticoids is an important adverse prognostic factor in newly diagnosed ALL patients [207]. Emerging evidence suggests that decreased expression of the BRG1, ARID1A, and SNF5 subunits appears to be associated

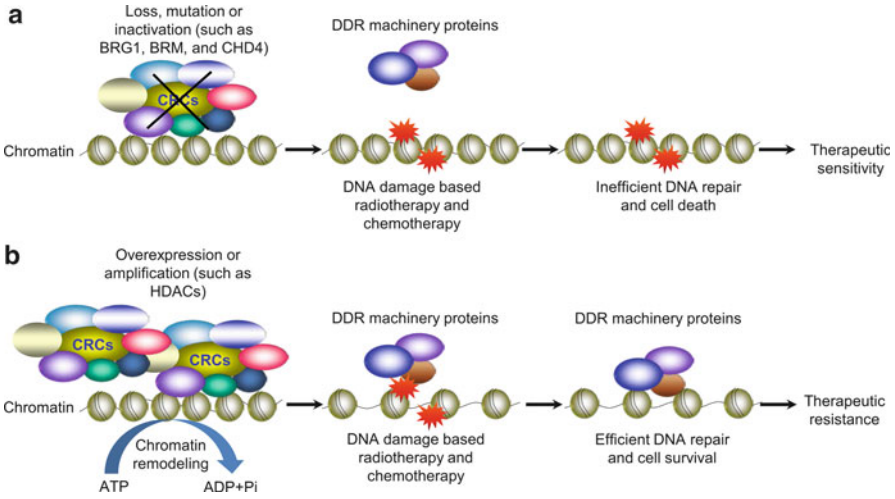

Fig. 16.2 Implication of CRCs in cancer therapeutics. Cancer cells with loss, mutation, or inactivation of the CRC components such as of BRG1, BRM and CHD4 are sensitive to DNA damage based radiotherapy and chemotherapy due to impaired DNA repair (**a**). In contrast, the CRC subunits such as HDACs are over expressed or amplified in cancer cells and promote efficient DNA repair, thus contributing to therapeutic resistance to DNA damage based radiotherapy and chemotherapy (**b**)

with glucocorticoid resistance in primary ALL cells [207]. Similarly, knockdown of BRG1 and BRM enhances cellular sensitivity to chemotherapy drug cisplatin by regulating efficient repair of the cisplatin DNA lesions [208]. Thus, cisplatin chemotherapy could be more effective in BRG1- and BRM-negative or inactivated tumors (Fig. 16.2a). Consistent with these findings, depletion of CHD4 renders cell significantly hypersensitive to DSB-inducing agents and PARP inhibitors as a consequence of impaired HR repair (Fig. 16.2a) [209]. As loss or mutation of BRG1, BRM and CHD4 has been observed in a variety of human cancers [186, 188, 210, 211], it is highly interesting to examine whether these tumors are sensitive to PAPR inhibitors or other DNA-damaging agents.

In contrast, overexpression of some components of CRCs is linked with therapeutic resistance (Fig. 16.2b). For instance, Rsf-1 overexpression confers paclitaxel resistance in ovarian cancer cells [212] and is associated with poor therapeutic response in rectal cancer patients treated with neoadjuvant chemoradiation therapy [191] and associated with incomplete response to radiotherapy in patients with nasopharyngeal carcinoma [192]. Notably, the Rsf-1-hSNF2H interaction is essential for developing resistance phenotype in tumors overexpressing Rsf-1 [212]. Thus, inhibition of Rsf-1 activity or disruption of the Rsf-1-hSNF2H interaction has the potential to sensitize cells to paclitaxel in human cancers with Rsf-1 amplication or overexpression. Similarly, HDAC2 is highly expressed in pancreatic ductal adenocarcinoma (PDAC) and confers resistance towards the topoisomerase II inhibitor etoposide in PDAC cells [201]. Consistently, selective inhibition of HDACs synergises with etoposide to induce apoptosis in PDAC cells [201]. In a broader

perspective, targeting the CRC-mediated DNA repair pathways might provide unique potential therapeutic avenues for human cancers when used in combination with DNA-damaging chemotherapeutic drugs [213, 214].

16.7 Conclusions and Perspectives

During the past decades, it has been made great progress in our understanding of the functional roles for ATP-dependent CRCs in transcription and DDR, and it has been increasingly recognized that these CRCs show remarkable diversity and specifity in their contributions to these biological processes. However, it remains unknown why the transcription and DDR pathways need multiple CCRs and whether or how these CRCs exert their functions in these processes in an integrated manner at molecular levels. In addition, the detailed mechanisms by which these CRCs regulate transcription and DDR and drive tumorigenesis and progression are largely unclear.

From a translational perspective, the importance of the CRCs in cancer causation and progression provides new avenues to improve cancer management by targeting the chromatin remodeling machinery. One example is that the CRCs predominantly function in the DNA repair pathways, which may contribute to therapeutic resistance in patients with cancers by enabling cancer cells to survival DNA damage induced by chemotherapeutic agents and radiotherapy [214]. Thus, targeting the CRC components and related DNA repair signaling pathways in human cancers could be efficacious as monotherapy or in combination with DNA-damaging agents [213, 214]. A case in point is the HDACs, whose inhibitors are emerging as promising drugs for cancer therapy that selectively kill cancer cells and sensitize cancer cells to DSB-inducing agents [200]. On the other hand, as components of the CRCs are frequently mutated in human cancers, this unique property of cancer cells gives a great opportunity to screen appropriate patients in clinic for optimum personal therapy using DNA-damaging radiotherapy and chemotherapy. Together, further work that directs to understand the in vivo function and mechanism of action of these CRCs will definitely provide opportunities to discover new therapeutic targets and therapeutic strategies for the treatment of cancer as well as other CRC-related diseases.

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