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The essential transcriptional function of BRD4 in acute myeloid leukemia cells

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Abstract

Acute myeloid leukemia (AML) is often initiated by genetic alterations of machineries that regulate chromatin and transcription, thereby blocking cell differentiation. Such mechanisms can also render leukemia cells vulnerable to perturbations of transcriptional regulators, which includes small-molecules targeting the coactivator protein BRD4. Numerous studies have validated BRD4 as a therapeutic target in diverse subtypes of AML, however the vital function of BRD4 in this disease is only beginning to be understood. Here we discuss the recent progress in elucidating the transcriptional function of BRD4 in AML cells, with an emphasis on the desirable attributes, but also the inherent limitations, of targeting general coactivator proteins as cancer therapy.

Introduction

Chromatin alterations are intimately linked with the control of transcription, and such processes are commonly deregulated in the pathogenesis of cancer (Dawson and Kouzarides 2012). This is highlighted by many large-scale cancer genome studies, which have identified frequent mutations in the genes encoding chromatin regulators during tumor evolution (Garraway and Lander 2013). While the majority of mutations in chromatin regulators found in cancer are predicted to be loss-of-function, a smaller subset of these mutations result in a gain-of-function that drives oncogenic gene expression. Thus, chromatin regulators represent viable targets for direct therapeutic intervention in cancer (Shen and Vakoc 2015).

Acute myeloid leukemia (AML) was one of the first malignancies in which genes encoding chromatin regulators were identified as drivers of cell transformation, and therefore AML serves as a paradigm for studying chromatin deregulation in cancer. As an example, the histone H3 lysine 4 methyltransferase protein MLL (also known as KMT2A) is often altered in AML via chromosomal translocations or mutations, thereby deregulating chromatin to cause a blockade in myeloid maturation. Other commonly mutated genes in AML include *DNMT3A* or *TET2*, which encode enzymes involved in DNA methylation and de-methylation, respectively. Genetic alterations of transcription factors (TFs) are also common in AML and lead to indirect alterations of chromatin structure and function to initiate disease progression.

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The widespread involvement of aberrant chromatin states in AML provides a rationale to explore the use of chromatin modulating therapeutics in this disease. One of the first examples of such an agent is 5-azacytidine, which inhibits DNA methyltransferases. 5-azacytidine and other related molecules are approved therapies in AML management, despite uncertainty regarding the mechanisms underlying their therapeutic efficacy. A major effort in the field in recent years has been to uncover other epigenetic vulnerabilities in AML, which has culminated in recently initiated clinical trials evaluating inhibitors of histone modifying enzymes (DOT1L and LSD1) and inhibitors of the chromatin reader protein BRD4. Here, we will review the current understanding of BRD4 as therapeutic target in AML.

BRD4 is a dependency and a therapeutic target in AML

BRD4 can be classified as a chromatin reader protein, as it contains two tandem bromodomain modules that engage in acetyl-lysine recognition to tether BRD4 to chromatin. BRD4 can also be classified as a transcriptional coactivator, as it can bind directly (or indirectly) to TFs to promote downstream effects on RNA polymerase II (Pol II) activation. Most of the available evidence suggests that BRD4 functions as a scaffold protein that links acetylated histones and TFs with the local assembly of the transcriptional apparatus at specific cis-regulatory elements. It was recently proposed that BRD4 also employs an intrinsic kinase and acetyltransferase activities, although the functional relevance of these activities to the transcriptional function of BRD4 remains to be validated. Studies over the past 16 years have implicated BRD4 in numerous cellular processes, including the regulation of transcription, DNA replication, and DNA repair.

While it might have been assumed that BRD4 performs a ‘general’ or ‘housekeeping’ role in various genomic processes, several groups identified small-molecule inhibitors of BRD4 bromodomains (and its closest homologs BRD2/BRD3/BRDT) in cell based screens evaluating for discrete biological effects (reviewed in X). These small-molecules were found to bind to the bromodomains in a competitive manner with acetyl-lysine, and hence result in the release of BRD4 and its homologs (which are often referred to as the bromodomain and extraterminal domain-containing proteins, or BET protein family) from cellular chromatin. Since the description of these molecules in 2010, numerous studies have demonstrated that chemical inhibition of BET proteins results in gene-specific effects on transcription, which are distinct from global transcription inhibition (Shi and Vakoc 2014). The specificity of transcriptional and cellular effects of BET inhibition have also allowed an investigation of disease-specific roles of BRD4, particularly in cancer.

In 2011, several independent groups demonstrated that BET inhibitors exhibit anti-AML activity in animal models of this disease. One of these studies was from our own laboratory, in which we identified BRD4 using a short-hairpin RNA (shRNA) screen that evaluated the essentiality of epigenetic regulators for disease progression in a mouse model of AML initiated by the MLL-AF9 and $Nras^{G12D}$ oncogenes. This screen identified BRD4 as a top vulnerability in this disease, based on the overall potency of the growth-arrest phenotype observed following BRD4 knockdown. Notably, a modest 4-fold knockdown of BRD4 is sufficient to trigger terminal differentiation and apoptosis in AML cells, while BRD4

knockdown only elicited cytostatic effects in other immortalized cell types. Through a collaboration with James Bradner's laboratory, we obtained the BET inhibitor JQ1 and showed that the phenotypic consequences observed upon BRD4 knockdown were faithfully recapitulated following exposure to nanomolar concentrations of this compound. The close similarity in the effects of BRD4 knockdown and JQ1 exposure at the phenotypic and global transcriptional level provided strong support that BRD4 was the relevant target underlying the effects of JQ1 in this cell type. Most notably, intra-peritoneal injection of JQ1 into AML-bearing animals was shown to delay disease progression in vivo at doses that exhibited minimal effects on normal hematopoiesis. While our mechanistic understanding of these effects was limited during the early stages of this research, two observations were made at this time would later shape our mechanistic evaluation of BRD4 in AML. First, the sensitivity to JQ1 was identified in MLL-rearranged subtype of AML, but was equally apparent in the other diverse genetic subtypes of this disease. Second, JQ1 caused a rapid, and pronounced, suppression of c-Myc transcription in AML cells, while Myc expression was unaffected by JQ1 in immortalized fibroblasts. Collectively, these experiments led us to propose that BRD4 is a novel epigenetic target in AML, and reinforced the utility of genetic screening to nominate therapeutic opportunities in cancer.

An independent study from the lab of Tony Kouzarides published at the same time also demonstrated the efficacy of BET inhibitors in the MLL-rearranged subtype of AML. In this study, a series of proteomic screens performed in AML nuclear lysates had found BET proteins in association with the transcription elongation regulators PAF and P-TEFb, which are known interacting partners of MLL-fusion oncoproteins. These findings motivated an evaluation of the BET inhibitor I-BET, which inhibits BET bromodomains in a similar manner to JQ1, in various pre-clinical models of the MLL-fusion subtype of AML. IBET was shown to suppress AML progression in these models, in association with transcriptional suppression of MYC, BCL2, and CDK6. While it still remains unclear whether BET proteins are needed for the function of MLL-fusion protein, this work provided strong evidence supporting BET proteins as therapeutic targets in AML. The lab of Robert Sims at Constellation Pharmaceuticals also showed independently that human AML cell lines (in vitro and well transplanted into mice) are sensitive to JQ1 in association with transcriptional suppression of *MYC*. Collectively, these convergent studies demonstrated that diverse subtypes of AML are vulnerable to BRD4 inhibition, and set the stage for evaluation of BET inhibitors as AML therapy in early stage clinical trials.

Starting in 2013, several independent groups initiated clinical studies of BET inhibitors in human cancer patients. The first Phase I study of a BET bromodomain inhibitor (called OTX-015) in leukemia patients was published in 2016. This dose escalation study evaluated OTX-015 in relapsed-refractory acute leukemia patients. This study provided the first glimpse into the toxicities of BET inhibition in humans, which includes reduced platelet counts, gastrointestinal symptoms, fatigue, and altered levels of coagulation factors, and led to the identification of suitable doses for future clinical investigation. Clinical responses were identified in a subset of patients treated on this trial at doses that were below the maximum tolerated dose, with a subset of patients achieving a complete remission. However, all of these patients eventually relapsed while receiving therapy. While this study provides initial support for BET inhibition as a therapeutic strategy in AML, numerous

challenges remain in identifying predictive correlates of BET inhibitor sensitivity and to determine optimal combination drug regimens to make responses more durable. It should be emphasized that sensitivity to BET inhibition is not limited to AML, but has also been established in lymphoid neoplasms, including lymphoma, B- and T-cell leukemias, multiple myeloma, and various solid tumors, which are indications under investigation in clinical trials.

One of the clear challenges to BET inhibitor therapy in AML will be the rapid emergence of drug resistant cells. Recent studies have dissected the molecular mechanisms of resistance BET inhibition in AML mouse models, which seem to implicate non-genetic mechanisms (ie, resistance mutations of BRD4 or other genes are not selected for). Instead, it appears that cells with reprogrammed transcriptional states can be selected for in the presence of BET inhibition, with are able to bypass the essential function of BRD4 to sustain growth-promoting genes, such as Myc. One of these BRD4-bypass pathways is via the WNT signaling pathway, and suggests that dual targeting BRD4 and WNT by lead to durable therapeutic responses. Several studies have now identified effective BET inhibitor drug synergies have also been identified, including combinations an enhanced synergy when combining BET inhibitors with FLT3 kinase inhibitors and cells harboring activating mutations of FLT3. This study also suggests that resistance to FLT3-inhibition might be overcome by targeting BET proteins. In the MLL-fusion subtype of AML, co-inhibition of BRD4 and DOT1L can also lead to synergistic anti-leukemia responses. These studies highlight how an expanding body of basic and translational research strategies can inform how BET inhibitors proceed in future clinical trials.

Despite the speed in which BET inhibitors have moved into clinical evaluation, it remains unknown why chemical inhibition of a general coactivator protein like BRD4 leads to therapeutic effects in AML. At the heart of this issue is the deficiency in our understanding of the leukemia maintenance function of BRD4, which has been the subject of intense investigation over the past five years. Below, we summarize our efforts, and those of other laboratories, in characterizing the essential transcriptional function of BRD4 in AML.

Hematopoietic lineage transcription factors use BRD4 as a cofactor

The central hypothesis in the field regarding the therapeutic index of BET inhibitors in cancer is that targeting BRD4 disproportionately suppresses cancer-promoting genes versus other gene classes. The mechanisms that underlie this gene-specificity are not well understood, but may relate to the manner in which BRD4 is recruited to chromatin. ChIP-seq analysis in murine MLL-AF9/Nras^{G12D}-transformed AML cells revealed that BRD4 tends to occupy acetylated nucleosomes, as well as nucleosome-free regions across genome. The presence of BRD4 at nucleosome depleted sites implicated the possibility that transcription factors were involved in this process. Indeed, prior work had implicated BET proteins in recognizing acetylated transcription factors GATA-1, NFkB, and Twist in other cellular contexts. Using an analysis of over-represented motifs at nucleosome-depleted sites in leukemia cells, we demonstrated that the TFs ERG, FLI1, PU.1, C/EBPα, C/EBPβ, and MYB are highly correlated with BRD4 in this cell type. Remarkably, each of these TFs are lineage-determinants which are vital for normal hematopoietic stem and progenitor cells.

Remarkably, ectopic expression of these TFs in heterologous cell types (fibroblasts) is will mobilize BRD4 to the unique binding sites observed in leukemia cells, thus demonstrating that TFs, either directly or indirectly, promote BRD4 recruitment to the genome in AML.

The mechanism by which these hematopoietic TFs stimulate BRD4 recruitment is via the use of the lysine acetyltransferase p300 as a coactivator, which prior work had shown can bind to each of these TFs. Genetic targeting and chemical inhibition of p300 in AML closely phenocopies the transcriptional effects of BRD4 knockdown, and leads to the release of BRD4 from chromatin. Not only is p300 recruited to chromatin by these TFs, but can also acetylate each of these TFs at functionally important sites. We characterized one of these sites on ERG in detail, which is a KGGK motif which can be acetylated by p300 one both lysines. The di-acetylated ERG peptide can bind directly to BRD4, and is likely to contribute to BRD4 tethering to the AML genome. However, it is likely that multiple TFs and histones associate with BRD4 via low-affinity interactions to account for its recruitment. Collectively, this study demonstrated the critical role of hematopoietic lineage TFs in employing BRD4 as a cofactor to promote transcriptional activation.

NSD3, CHD8, Mediator, and P-TEFb are effectors of BRD4 in AML cells

Another key aspect of BRD4 function in AML concerns the downstream mechanisms by which this regulator activates RNA polymerase II. Proteomic studies have repeatedly shown that BRD4 engages in numerous protein-protein interactions, and thus acts as a scaffold for other regulators. One consistent BRD4 interacting partner is the kinase complex P-TEFb, which consists of Cdk9 and a Cyclin subunit. P-TEFb phosphorylates the pausing factors DSIF and NELF, as well as RNA polymerase II itself to promote transcription elongation. Studies in AML and in various other cell types have shown that JQ1 promotes the rapid eviction of P-TEFb from chromatin in parallel with an accumulation of paused RNA polymerase at nearby genes. Moreover, CRISPR exon scanning experiments have revealed that the C-terminal motif of BRD4, which engages in a direct interaction with P-TEFb, is also vital for AML cell proliferation.

Multiple subunits of the Mediator complex can also bind to BRD4. While the MED23 subunit of Mediator appears to be required for the BRD4 interaction, the precise binding surface that links these two factors has yet to be mapped. In AML and in other cell types, BRD4 occupancy across the genome is tightly correlated with the Mediator complex, with JQ1 releasing Mediator complexes from chromatin. In addition, genetic targeting of certain Mediator subunits in AML cells, such as MED12, MED13, MED23, and MED24 will lead a similar differentiation phenotype and transcriptional alterations as observed upon targeting BRD4. Mediator is also known to regulate P-TEFb activity via its head module, which lead to the coordination, and perhaps synergy betwee, these two positive regulators of RNA polymerase II activity.

One of the defining domains of BET proteins is the extraterminal (ET) domain, which has been shown to activate transcription when artificially tethered to a minimal promoter. In AML cells, the ET domain physically associates with a short isoform of NSD3, which acts as an adaptor protein that couples BRD4 with the CHD8 chromatin remodeler. Notably,

genetic targeting of NSD3 or CHD8 leads to similar phenotypes and transcriptional effects as observed upon BRD4 inhibition. Collectively, these studies highlight how BRD4 utilizes several interacting partners to facilitate chromatin remodeling and transcriptional activation in AML cells.

While BRD4-containing protein complexes are broadly associated with active promoters and enhancers across the AML genome, JQ1 leads to the disproportionate eviction of these complexes from specific *cis* elements. This includes a distal cluster of enhancers (or super-enhancer) at the *Myc* locus, thus accounting for the hyper-sensitivity of *Myc* transcription to BRD4 inhibition in AML. Remarkably, the locations of severe BRD4 eviction following JQ1 exposure are highly correlated with genes having pro-leukemic functions, as revealed through gene ontology analysis. Moreover, these cis elements are skewed in having more Myb occupancy than the other hematopoietic TFs described above. This result implies that Myb is more suppressed by JQ1 than other hematopoietic TFs, which might be contributor to the overall sensitivity of leukemia cells to JQ1, since prior studies have shown that AML is more sensitive to Myb suppression than normal myeloid cells. These findings suggest that biased release of BRD4 complexes for specific cis elements is the major determinant of the gene-specific transcriptional effects of BET bromodomain inhibition.

One key question yet to be addressed is why some cis-elements are more sensitive than others to JQ1. One possibility is clustered enhancers (or super-enhancers) recruit BRD4 in a cooperative fashion, which is more vulnerable to chemical inhibition than sites lacking cooperative BRD4 recruitment. Another possibility is that many sites in the genome recruit BRD4 in a bromodomain-independent manner, and hence resist JQ1-mediated displacement. A third possibility is that specific *cis* elements exhibit dynamic ON-OFF rates of BRD4 recruitment, which consequently are preferentially evicted by JQ1 when compared to more static cis elements. Regardless of the underlying mechanism, the skewed pattern of BRD4 release from the genome from leukemia-relevant genes is clearest mechanism to date that accounts for the therapeutic index of BET inhibitors in AML.

Conclusions

Mechanistic studies of BRD4 in the MLL-fusion subtype of AML has unexpectedly revealed that this regulator is almost entirely engaged as a cofactor for hematopoietic lineage transcription factors, and not via an association with the MLL-fusion oncoprotein. Thus, the anti-leukemia activity of JQ1 occurs despite the MLL-fusion-mediated gene activation (e.g. at HOX gene clusters) is unperturbed. Thus, targeting BRD4 in AML may share more in common conceptually with targeting lineage-specific TFs in prostate (androgen receptor) and breast (estrogen receptor) cancers. However, it must be recognized that, unlike AR and ER examples, BRD4 is likely to function as a key coactivator in all human tissues, and hence the largest concern for BET inhibitors as therapeutics lies in their potential for undesirable toxicities, which is just beginning to be exposed in clinical trials. A key priority for future investigation will be to expose coactivator perturbations which are capable achieving potent anti-cancer activity, while minimizing the broad suppression of multiple TFs. In AML, this would best be achieved by targeting coactivators in a manner that suppresses Myb function, while preserving other vital TFs for normal hematopoiesis. Continued efforts aimed at

defining the fundamental interaction between TFs and coactivators might expose newer modalities to reprogram transcription in a manner suitable for therapeutic efficacy in cancer, but also in the numerous other diseases with a genetic basis.

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