The Essential Transcriptional Function of BRD4 in Acute Myeloid Leukemia

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Acute myeloid leukemia (AML) is often initiated by genetic alterations of machineries that regulate chromatin and transcription, thereby blocking cell differentiation. Such mechanisms may 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.

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). Although 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. In addition, unmutated chromatin regulators can support the function of other oncoproteins to drive tumorigenesis, thereby acting as nononcogene dependencies in cancer. Thus, cancer cells are often addicted to certain chromatin regulators, which represent viable targets for direct therapeutic intervention in this disease (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 (Greenblatt and Nimer 2014; Wouters and Delwel 2016). 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 (Krivtsov and Armstrong 2007). Other commonly mutated genes in AML include *DNMT3A* or *TET2*, which encode enzymes involved in DNA methylation and demethylation, respectively (Delhommeau et al. 2009; Ley et al. 2010). 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 (Rosenbauer and Tenen 2007).

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 (Silverman et al. 2002; Kantarjian et al. 2006; Issa et al. 2015). 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 (Bernt et al. 2011; Nguyen et al. 2011; Harris et al. 2012). Here, we will review our 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 acetyllysine recognition to tether BRD4 to chromatin (Wu and Chiang 2007). 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 (Shi and Vakoc 2014). 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. However, it was recently proposed that BRD4 employs intrinsic kinase and acetyltransferase activities,

although the functional relevance of these activities to the transcriptional function of BRD4 remains to be validated (Devaiah et al. 2012, 2016). Studies over nearly the past 20 years have implicated BRD4 in numerous cellular processes, including the regulation of transcription, DNA replication, and DNA repair (Wu and Chiang 2007; Floyd et al. 2013).

Although it might have been assumed that BRD4 performs a general role in regulating 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 Filippakopoulos et al. 2010; Nicodeme et al. (2010). These small molecules were found to bind to the bromodomains in a competitive manner with acetyllysine, 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 shown 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 has also allowed an investigation of disease-specific roles of BRD4, particularly in cancer.

In 2011, several independent groups showed that BET inhibitors exhibit anti-AML activity in animal models of this disease (Dawson et al. 2011; Mertz et al. 2011; Zuber et al. 2011b). 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 chromatin regulators for disease progression in a mouse model of AML initiated by the MLL-AF9 and *Nras*^{G12D} oncogenes (Zuber et al. 2011b). 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 fourfold knockdown of BRD4 is sufficient to trigger terminal differentiation and apoptosis in AML cells, whereas 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 (Zuber et al. 2011b). Most notably, intraperitoneal injection of JQ1 into AML-bearing animals was shown to delay disease progression in vivo at doses that showed minimal effects on normal hematopoiesis. Although our mechanistic understanding of these effects was limited during the early stages of this research, two observations were made at this time that would come to 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, whereas Myc expression was unaffected by JQ1 in immortalized fibroblasts. Collectively, these experiments led us to propose that BRD4 was a novel epigenetic target in AML and highlighted the utility of genetic screening to nominate therapeutic opportunities in cancer.

An independent study from the laboratory of Tony Kouzarides published at the same time also showed the efficacy of BET inhibitors in the MLL-rearranged subtype of AML (Dawson et al. 2011). 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 (Muntean et al. 2010; Yokoyama et al. 2010). These findings motivated an evaluation of the BET inhibitor I-BET, which inhibits BET bromodomains in a similar manner to JQ1, in various preclinical models of the MLL-fusion subtype of AML. I-BET was shown to suppress AML progression in these models, in association with transcriptional suppression of *MYC*, *BCL2*, and *CDK6* (Dawson et al. 2011). Although it is unlikely that BET proteins act directly to support the function of MLL-fusion proteins, this work provided strong evidence supporting BET proteins as therapeutic targets in AML. The laboratory of Robert Sims at Constellation Pharmaceuticals also showed independently that human AML cell lines are sensitive to JQ1 in association with transcriptional suppression of *MYC* (Mertz et al. 2011). Collectively, these convergent studies showed 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.

Clinical studies were initiated in 2013 to evaluate the effect 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 (Berthon et al. 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 hence led to the identification of suitable dosing regimens for future clinical investigation. Clinical responses were identified in a subset of patients treated on this trial at doses that were near or below the maximum tolerated dose, with a subset of patients achieving a complete remission. However, all of these patients showing a complete remission eventually relapsed while receiving therapy (Berthon et al. 2016). Although this study provides encouraging support for BET inhibition as a therapeutic strategy in human AML, numerous challenges remain in identifying predictive correlates of BET inhibitor sensitivity and in determining 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 (Cai et al. 2015).

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 have implicated nongenetic mechanisms (Fong et al. 2015; Rathert et al. 2015). Instead, it appears that cells with reprogrammed transcriptional states can be selected for in the presence of BET inhibition that 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 could lead to durable therapeutic responses (Rathert et al. 2015). Numerous studies have identified effective BET inhibitor drug synergies, including combinations of BET inhibitors with FLT3 kinase inhibitors in cells harboring activating mutations of FLT3 (Fiskus et al. 2014). 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 antileukemia responses (Gilan et al. 2016). 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 5 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 (Roe et al. 2015). 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, NF- κ B, and Twist in other cellular contexts (Lamonica et al. 2011; Shi et al. 2014). Using an analysis of overrepresented motifs at BRD4-occupied sites in leu-

kemia cells, it was found that the TFs ERG, FLI1, PU.1, C/EBP α , C/EBP β , and MYB are highly correlated with BRD4 in this cell type (Roe et al. 2015). Notably, each of these TFs are lineage determinants that are vital for normal hematopoietic stem and progenitor cells (Zhang et al. 1997; Fisher and Scott 1998; Spyropoulos et al. 2000; Hirai et al. 2006; Greig et al. 2008; Loughran et al. 2008). Ectopic expression of these TFs in heterologous cell types (fibroblasts) will reprogram BRD4 to the unique binding sites found in leukemia cells, which suggests that TFs, either directly or indirectly, promote BRD4 recruitment to the genome in AML (Roe et al. 2015).

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 (Dai et al. 1996; Parker et al. 1999; Yamamoto et al. 1999; Tomita et al. 2000; Erickson et al. 2001; Sano and Ishii 2001; Bai et al. 2005; Sandberg et al. 2005; Cesena et al. 2007; Li et al. 2011; Schlottmann et al. 2012; Roe et al. 2015). Genetic targeting and chemical inhibition of p300 in AML closely phenocopies the transcriptional effects of BRD4 knock-down and leads to the release of BRD4 from chromatin (Roe et al. 2015). Not only is p300 recruited to chromatin by these TFs, but it can also acetylate each of these TFs at functionally important sites (Tomita et al. 2000; Erickson et al. 2001; Bai et al. 2005; Cesena et al. 2007; Schlottmann et al. 2012; Blee et al. 2016). We characterized one of these sites on ERG in detail, which is a KGGK motif that can be acetylated by p300 on both lysines. The diacetylated 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, our study showed the critical role of hematopoietic lineage TFs in using BRD4 as a cofactor to promote transcriptional activation in AML.

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 BRD4-interacting partner is the kinase complex P-TEFb, which consists of Cdk9 and a cyclin subunit (Jang et al. 2005; Yang et al. 2005). P-TEFb phosphorylates the pausing factors DSIF and NELF, as well as RNA polymerase II itself to promote transcription elongation (Peterlin and Price 2006). 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 (Loven et al. 2013; Bhagwat et al. 2016). Moreover, CRISPR exon scanning experiments have revealed that the carboxy-terminal motif of BRD4, which engages in a direct interaction with

P-TEFb, is also vital for AML cell proliferation (Bisgrove et al. 2007; Shi et al. 2015).

Multiple subunits of the Mediator complex can also bind to BRD4 (Jiang et al. 1998; Donner et al. 2010). Although 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 (Wang et al. 2013). 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 (Donner et al. 2010; Loven et al. 2013; Bhagwat et al. 2016). In addition, genetic targeting of certain Mediator subunits in AML cells, such as MED12, MED13, MED23, and MED24 will lead to a similar differentiation phenotype and transcriptional alterations as observed upon targeting BRD4 (Bhagwat et al. 2016). Mediator is also known to regulate P-TEFb activity via its head module (Conaway and Conaway 2013), which leads to the coordination, and perhaps synergy, between these two positive regulators of RNA polymerase II activity (Allen and Taatjes 2015).

One of the defining domains of all four BET proteins is the extraterminal (ET) domain, which has been shown to activate transcription when artificially tethered to a minimal promoter (Shen et al. 2015). 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 (Shen et al. 2015). Notably, genetic targeting of NSD3 or CHD8 leads to similar phenotypes and transcriptional effects as observed upon BRD4 inhibition (Shen et al. 2015). Collectively, these studies highlight how BRD4 uses several interacting partners to facilitate chromatin remodeling and transcriptional activation in AML cells.

Although 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 (Loven et al. 2013; Shi et al. 2013). This includes a distal cluster of enhancers (or super-enhancer) at the *Myc* locus, thus accounting for the hypersensitivity of *Myc* transcription to BRD4 inhibition in AML (Shi et al. 2013; Bhagwat et al. 2016). 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 (Bhagwat et al. 2016). Moreover, these *cis* elements are skewed in having more Myb occupancy than the other hematopoietic TFs described above (Bhagwat et al. 2016). This result implies that Myb is more suppressed by JQ1 than other hematopoietic TFs, which might be a contributor to the overall sensitivity of leukemia cells to JQ1, because prior studies have shown that AML cells are more sensitive to Myb suppression than normal myeloid cells (Anfossi et al. 1989; Calabretta et al. 1991; Zuber et al. 2011a). These findings suggest that biased release of BRD4 complexes for specific *cis* elements is a key 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 pos-

sibility is that clustered enhancers recruit BRD4 in a cooperative fashion, which is more vulnerable to chemical inhibition than sites lacking cooperative BRD4 recruitment (Loven et al. 2013). 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 show highly dynamic ON–OFF rates of BRD4 recruitment, which consequently are preferentially evicted by JQ1 when compared with more static *cis* elements. Regardless of the underlying mechanism, the skewed pattern of BRD4 release from the genome from leukemia-relevant genes is the clearest mechanism to date that accounts for the therapeutic index of BET inhibitors in AML.

CONCLUSION

Mechanistic studies of BRD4 in the MLL-fusion subtype of AML have 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 (Roe et al. 2015). Thus, the antileukemia activity of JQ1 occurs despite the output of MLL-fusion oncoproteins being 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 (Bhagwat and Vakoc 2015). 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 (Amorim et al. 2016; Berthon et al. 2016). A key priority for future investigation will be to expose coactivator perturbations that are capable of achieving potent anticancer 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 improved modalities to reprogram transcription in a manner suitable for therapeutic efficacy in cancer.

ACKNOWLEDGMENTS

C.R.V. is supported by the Leukemia and Lymphoma Society, the Burroughs-Wellcome Fund, the Pershing Square Sohn Cancer Research Alliance, the Starr Cancer Consortium, and the National Institutes of Health (NIH)/National Cancer Institute (NCI) grants RO1 CA174793 and 5P01CA013106.

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Cold Spring Harbor Symposia on Quantitative Biology

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Cold Spring Harb Symp Quant Biol 2016 81: 61-66 originally published online February 7, 2017
Access the most recent version at doi:[10.1101/sqb.2016.81.031039](https://doi.org/10.1101/sqb.2016.81.031039)

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