Short Hairpin RNA Screen Reveals Bromodomain Proteins as Novel Targets in Acute Myeloid Leukemia

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Targeting chromatin regulators for the treatment of malignancies has shown great promise, but also revealed significant challenges. By employing an elegant shRNA screen and a selective pharmacological inhibitor, a recent study published in *Nature* establishes the bromodomain protein Brd4 as novel target in acute myeloid leukemia (AML).

Short hairpin RNA screens to identify critical regulators of cancer cell growth are a promising methodology but have been technically challenging. In particular, such screens can be biased by selections introduced when shRNAs are introduced into cells. The recent paper by Zuber and colleagues combines a series of critical variations on the approach to identify the bromodomain protein Brd4 as a regulator of c-Myc and a viable therapeutic target for acute myeloid leukemia (AML) (Zuber et al., 2011b). A critical element in the screen is the use of vectors that place shRNAs on a regulatable promoter to avoid selection bias (Zuber et al., 2011a). Other important features of the study are the well-defined biologic system, in vivo confirmation of selected targets, and the serendipitous ability to confirm the results with the recently described Brd4 inhibitor JQ1 (Filippakopoulos et al., 2010). This combination of elements should stimulate a renewed and better-educated interest in short hairpin screens for identifying targets for cancer therapy.

Genomic alterations, along with the subversion of epigenetic pathways, can lead to AML, a particularly aggressive form of leukemia with relatively few treatment options. Zuber et al. developed a strategy designed to specifically inhibit the growth of leukemic cells by inhibiting known regulators of chromatin function. A circumscribed custom library of ~1100 shRNAs against 243 known chromatin regulators driven from an inducible vector was introduced into murine leukemic cells coexpressing MLL-AF9 and an activated form of Nras (G12D). Transduced cells were cultured for two weeks under conditions allowing the expression of shRNAs. Subsequent profiling of shRNAs by deep sequencing identified shRNAs that were underrepresented and whose targets thus deemed essential for leukemic growth. The shRNA-targeting the Brd4 gene was among the most depleted shRNAs identifying this gene as important for AML cell survival. These findings were further carefully validated using a series of in vitro and in vivo experiments. The investigators surmised an essential role for the gene encoding the proto-oncoprotein, c-Myc, since Brd4 was previously shown to regulate c-Myc expression. Moreover, c-Myc has long been recognized as a regulator of leukemia biology and has been suspected to play a role in the renewal of leukemic stem cells. However, attempts to target the protein therapeutically have been unsuccessful. Hence, c-Myc emerges yet again as a critical nodal point in the pathogenesis of leukemias, but its regulation by Brd4 leads to a novel approach to downregulating c-Myc by targeting a chromatin-associated protein that regulates its expression.

Bromodomain proteins are a family of transcriptional regulators implicated in regulating epigenetic memory. Brd4 is a member of the BET family of proteins that is characterized by the presence of two tandem bromodomains, the only known direct recognition modules of acetylated lysines. Brd4 interacts with P-TEFb and functions as a global transcriptional coactivator (Wu and Chiang, 2007; Zeng and Zhou, 2002). Brd4 is recruited to acetylated histones and stimulates transcriptional elongation, but recent results suggest the protein may play a role in a variety of chromatin regulating complex (Rahman et al., 2011). Mixed lineage lymphoma (MLL) fusion proteins of the type used to generate the animal model studied by Zuber and colleagues also regulate elongation, and it is tempting to speculate that elongation of c-Myc mRNA is a critical Brd4-dependent event, but further experimentation is necessary to test this hypothesis.

In a remarkable convergence of initially distinct lines of research, BET proteins were recently identified as targets of the related compounds JQ1 and I-BET (Filippakopoulos et al., 2010; Nicodeme et al., 2010). Both are first generation, cell-permeable small molecules that bind competitively to the acetyl-lysine recognition motifs with high specificity for the bromodomains of BET family members but not other chromatin-associated proteins. BET bromodomains are structurally well characterized and display well-defined modes of acetyl-lysine binding, making them attractive drug targets when the proteins, as here, have a demonstrated biologic role in disease maintenance. Zuber et al. showed that JQ1 treatment of numerous leukemic cell lines involving MLL and non-MLL translocations, primary leukemic cells, as well as mice bearing MLL-AF9/Nras G12D leukemic cells leads to leukemic cell death, myeloid differentiation and, in the

case of the mice, extended survival, thus phenocopying the effects of Brd4 knockdown and further defining Brd4 as an Achilles heel in a wide range of leukemias. JQ1 and I-BET bind all examined BET family proteins. It is therefore possible that derivatives of these compounds could be developed that uniquely bind only one of the tandem bromodomains or distinguish between BET family proteins to increase their therapeutic value. Together with previous work on BET inhibitors, this study impressively demonstrates that contrary to widespread views, proteins that lack intrinsic enzymatic activity and function instead as adaptor molecules can represent powerful drug targets. Chromatin-associated proteins are especially attractive candidates for pharmacological intervention since some histone modifications carry the potential for epigenetic memory. Hence, therapeutic benefits might persist beyond the duration of the treatment.

An unanswered question raised by these studies is why JQ1 demonstrates antitumor activity without apparent toxicity. As noted, JQ1 interacts with all examined BET proteins, many of which are essential for development (Houzelstein et al., 2002). Moreover, Brd4 is widely expressed and associates with a host of genes (Rahman et al., 2011). The answer likely relates to differential sensitivities to partial Brd4 inhibition/depletion between highly proliferative cells and normal, slow growing, or resting cells. The c-Myc gene, which is bound by Brd4, is dynamically regulated in part at the level of transcriptional elongation. Brd4 associates with the transcription elongation complex, P-TEFb, presumably mediating rapid transitions in transcription elongation (Jang et al., 2005; Yang et al., 2005). Limiting the levels of Brd4 or its association with acetylated chromatin might lower productive c-Myc transcription, thus slowing the growth of rapidly dividing cells. Similar molecular mechanisms might account for the anti-inflammatory effects of I-BET. Interestingly, a separate recent study suggests that Brd4 may be a target for therapy in a rare disease, NUT midline carcinoma (NMC) (Filippakopoulos et al., 2010). Taken together, these observations lead one to speculate that an increased dependence on Brd4 may be a common feature of malignant cells compared to untransformed cells. Why this is so and whether Brd4 targeting molecules will have a function in therapy for other malignancies will be an important area for future studies.

It is a profound curiosity in science that "what goes around, comes around," or, in other words, important observations have a way of repeating themselves. In a second study by Zuber and colleagues, the group used a similar short hairpin approach to identify target genes for therapy in AML and identified the c-Myb oncogene as a therapeutic target for AML. The two studies raise striking echos of the 1989 article by Anfossie, Gewirtz and Calabretta stating that c-Myb antisense RNA induced death of AML cells, whereas c-Myc antisense oligomers induced differentiation of cells with a less profound induction of cell death (Anfossi et al., 1989). Thus, although our technology moves forward, it would seem that biology still points us towards the critical nodes of transformation initially identified by the study of viral oncogenes and chromosomal translocations.

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