Editorial

Stem Cell INVESTIGATION

Pharmacological inhibition of aberrant transcription factor complexes in inversion 16 acute myeloid leukemia

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Within the hematopoietic system, core binding factor beta (CBFβ) normally forms a complex with the master hematopoietic regulator, RUNX1 (1) and stabilizes its binding to DNA (2). In core binding factor (CBF) acute myeloid leukemias (AMLs), chromosomal rearrangements alter either *RUNX1* or *CBFβ*, thus dysregulating normal hematopoiesis. These AMLs are classified as having a favourable prognosis with complete remission expected following cytarabine and anthracycline based chemotherapy (3). However, relapse rates remain high and durable responses to salvage chemotherapy remain uncommon, therefore there is still an urgent need for new therapies.

The inversion 16 (inv(16)) CBF AML subtype does not involve the *RUNX1* gene directly, but arises as a consequence of the expression of an abnormal CBFβ-SMMHC (core binding factor beta-heavy chain of smooth muscle myosin) fusion protein (4) causing a block in hematopoietic differentiation (5). CBFβ-SMMHC can act in a dominant negative manner to CBFβ and interacts with RUNX1 with 10 fold higher affinity due to the presence of the High Affinity Binding Domain at the N-terminus of SMMHC. It was therefore originally thought that CBFβ-SMMHC simply sequesters RUNX1 and stops its binding to DNA. However, this was refuted by an inv(16) knock-in mouse model which showed that RUNX1 activity was crucial to leukemogenesis as knockout of RUNX1 prevented the expected CBFβ-SMMHC mediated differentiation block (6).

Initial insights into the action of CBFβ-SMMHC came from a genome-wide analysis of its binding sites in an inv(16) cell line and a patient sample, which found that RUNX1 appeared to be co-localizing with the fusion protein at its target genes (7). After *CBFβ-SMMHC* knockdown by shRNA, cells were found to down-regulate genes associated with a stem cell phenotype and self-renewal and up-regulate more differentiated myeloid cell related genes, indicating that the fusion protein was directly responsible for the differentiation block. Pulikkan and colleagues (8) have taken this work further by exploring the mechanisms of growth deregulation in this type of AML by pharmacologically inhibiting the CBFβ-SMMHC:RUNX1 complex directly.

The inhibition of transcription factors has long been thought to be a holy grail in therapeutics as these molecules were considered unsuitable for drug targeting. The few exceptions include natural ligands, such as All-Trans Retinoic Acid (ATRA) in Acute Promyelocytic Leukemia which was a first example of a small molecule compound targeting an aberrant transcription factor, PML-RARA. The authors of the Pulikkan *et al.* study, from the groups of Lucio Castilla and John Bushweller (8), had previously identified inhibitors from a pharmacological screen using a fluorescence resonance energy transfer (FRET) assay based on blocking the interaction between RUNX1 and CBFβ-SMMHC. Using further modifications they made the compound highly selective for CBFβ-SMMHC:RUNX1, with the most effective molecule being AI.10.49 (9). They showed that inv(16) AML had marked overexpression of *MYC* and that after treatment with their compound, *MYC*

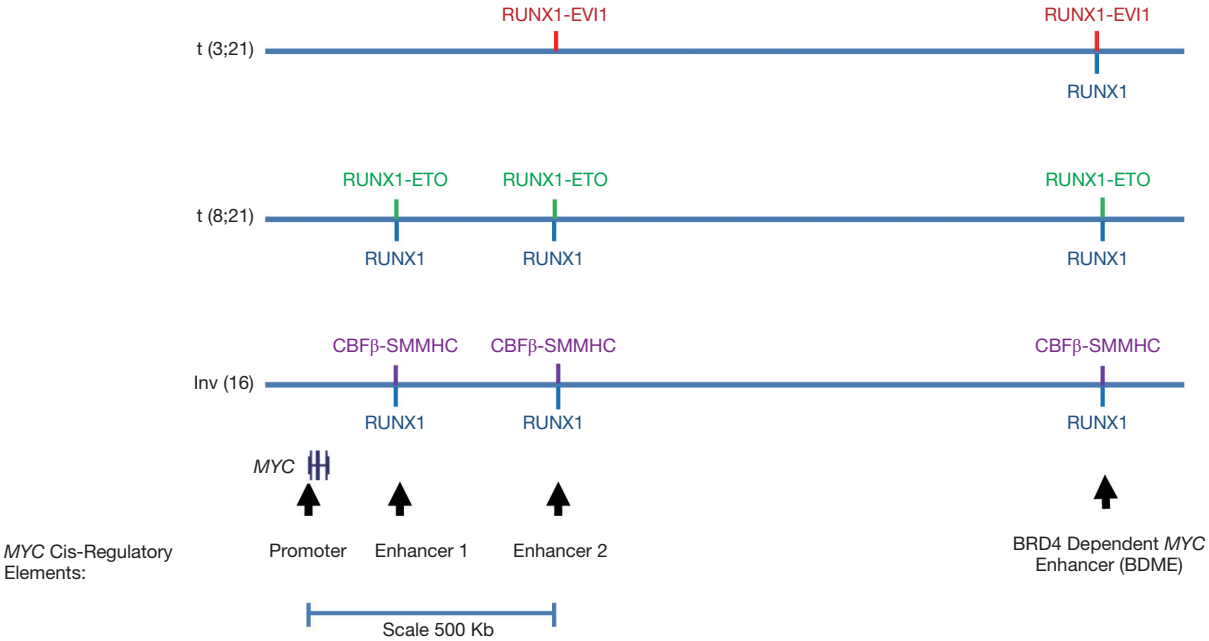

Figure 1 Binding sites of aberrant fusion proteins and RUNX1 around the *MYC* locus in core binding factor AMLs. AMLs, acute myeloid leukemias.

was one of the most down-regulated genes. Furthermore, knockdown of *MYC* using RNA interference conferred a significant survival advantage in xenotransplantation experiments and this recapitulated the phenotypic effects of AI.10.49. However, the mechanism by which *MYC* is overexpressed in inv(16) AML was previously not known.

MYC is well known to be overexpressed across many types of AML (10) and overexpressing *MYC* in normal hematopoietic progenitors led to the development of AML in mouse models (11). The mechanism of how *MYC* is overexpressed is understood in *NPM1* mutated AML; here NPM1c inhibits members of the E3 ligase family that normally promote the proteasomal degradation of the MYC protein (12). In addition, CEBPA normally negatively regulates *MYC* via an E2F binding site in the *MYC* promoter and consequently in *CEBPA* mutated AML, this repression is relieved (13). Finally, secondary activating mutations in signaling receptors such as FLT3-ITD and cKIT can upregulate *MYC* by STAT5 signaling (14). Inv(16) deploys an altogether different mechanism.

Pulikkan and colleagues (8) showed by chromatin immunoprecipitation (ChIP) assays that RUNX1 normally binds to 3 downstream *MYC* enhancer elements and that use of their inhibitor increases RUNX1 binding at this position, as well as globally. Furthermore, they showed by a chromatin conformation capture assay that these three enhancer elements directly interact with the *MYC* promoter and each other and that treatment with the compound strengthened these interactions. Functionally, they showed that deletion of any of these enhancers by CRISPR-Cas9 gene editing reduced *MYC* transcription and cell viability. The most distal of these three enhancers has been previously characterised as a BRD4-mediated *MYC* enhancer (BDME) (15) (*Figure 1*). In defining the molecular details of how the compound interferes with aberrant *MYC* regulation, Pulikkan *et al.* (8) showed that BRD4 knockdown by RNA interference in the inv(16) cell line reduced *MYC* transcripts levels. They went on to show synergism between the BRD4 inhibitor JQ1 (16) and their inhibitor AI.10.49 *in vitro* whereby cell viability was decreased and *in vivo* whereby xenotransplanted mice survived for much longer.

These results raise exciting therapeutic prospects as the combination of JQ1 and AI.10.49 could have a role in the relapsed/refractory setting or frontline in those patients too frail for intensive chemotherapy. However, there will need to be several further developments before such drugs may be suitable for therapeutic use. The half-life of AI.10.49 is 380 minutes in mice (9) and needs to be increased to be suitable for humans. The half-life of JQ1 is also very short at

around 1 hour (16), but there have been several attempts to produce modified BET inhibitors; OTX001 looks like one of the most promising with a half-life of 6 hours in humans, making it suitable for oral dosing four times a day, and with several clinical responses reported in case series (17).

Another important question relates to whether this therapy will work on quiescent pre-leukemic and leukemic stem cells where *MYC* expression may be low (18). It is increasingly recognized that such cells may survive intensive chemotherapy and may act as a reservoir of cells for relapse (19). Whilst it is thought that pre-leukemic and leukemic stem cells are aberrantly regulated by the CBFβ-SMMHC fusion protein as the inv(16) translocation is a first hit mutation in AML, it remains to be seen whether the presence of the fusion protein is sufficient to de-repress *MYC* transcription.

Shi and colleagues (15) showed that in a MLL-AF9 AML context, BRG1, an ATPase member of the SWI/SNF complex was key to establish and open chromatin conformation at the BDME enhancer. In inv(16) AML, BRG1 binds not only to this enhancer but also two other RUNX1-binding enhancers and the *MYC* promoter and use of AI.10.49 reduces BRG1 to all of these cis-regulatory elements (8). Furthermore, during hematopoietic differentiation RUNX1 has previously been described as acting as a repressor by recruiting RING1B, a member of the polycomb-repressive complex (PRC) (20). RING1B binding to each of the three downstream *MYC* enhancers was shown to be increased after AI.10.49 treatment and in a time course experiment following the addition of AI.10.49, it was demonstrated that as BRG1 binding decreased, RING1B binding increased (8). Consequently there appears to be competition between active transcriptional complexes including BRG1 and the gene silencing machinery including RING1B and the use of AI.10.49 pushes this equilibrium towards repression.

The finding that RUNX1 replaces BRG1 at active enhancers with RING1B, a member of the PRC, confirms multiple reports showing that RUNX1 can act as a transcriptional repressor in both normal and abnormal hematopoiesis (1,21). In our work we also found an interplay between RUNX1 and another CBF fusion protein, RUNX1-ETO, the product of the t(8;21) translocation. RUNX1 dynamically binds to a crucial cis-regulatory element of the Cyclin D2 (*CCND2)* gene and inhibits its transcription, thus regulating the cell cycle at the G1/S checkpoint (22). In t(8;21) AML, the RUNX1-ETO fusion protein competes with RUNX1 for binding an upstream element and cooperates with the AP-1 transcription factor family in binding to the *CCND2* promoter, thus leading to a de-repression of transcription. In the context of the *MYC* enhancers, it would be very interesting to know which other proteins are present at the active *MYC* enhancers which cooperate to drive *MYC* transcription. Mandoli and colleagues showed that globally at CBFβ-SMMHC:RUNX1 binding sites that GATA and ETS factors such as FLI1, PU.1 and ERG bind (7) but the full composition of the factor complex at *MYC* enhancers is unknown.

Fusion protein interference with RUNX1-mediated *MYC* regulation may also play also a role in other well-characterised types of CBF AML, such as the t(8;21) and the t(3;21) which fuses the RUNX1 DNA-binding domain to the EVI1 transcriptional repressor. *Figure 1* shows the binding locations of these complexes as well as of RUNX1 to *MYC* cis-regulatory elements based upon ChIP-seq data from the Mandoli and colleagues study (7) and our own published work (23,24). Consequently, when *RUNX1-ETO* was depleted by the use of siRNA, we found a decrease in *MYC* transcript levels.

The inhibition of protein complex formation provides an intriguing way of treating cancer and requires a significant structural and biological understanding of the proteins involved. Another inhibitor from the Bushweller lab allosterically alters the binding of key CBFβ residues to RUNX1 and hence decreases RUNX1 binding at its target sites and could be used to exploit the dependency of CBF AML on wild-type RUNX1 (25). This inhibitor had efficacy on a variety of AML cell lines including Kasumi-1, which is driven by the t(8;21) translocation but remarkably had minimal effect on normal hematopoiesis. Another example where such a strategy may be employed is in NPM1 mutated AML, which is the most common driver mutation in karyotypically normal AML. NPM1 is required for the cytoplasmic localization and destabilization of Fbw7γ in order to prevent nuclear degradation of several crucial AML oncoproteins such as MYC, NOTCH, CYCLIN E and JUN (12). A recurrent protein-interacting domain in NPM1 has been identified which could be therapeutically targeted (26). Furthermore, in inv(16) AML, there is a 28 amino acid Assembly Competence Domain near the C-terminus which is involved in the oligomerization of the CBFβ-SMMHC protein and this interface could potentially be targeted to prevent the formation of oligomeric complexes. Such a strategy has already been carried out in t(8;21) AML where a polypeptide (NC128) was used to inhibit oligomerization

of RUNX1-ETO via the Nervy Homology Region 2 (27).

One further major approach to therapeutics has been the coupling of molecules which recruit an E3 ubiquitin ligase to small molecules which can bind a transcription factor. This allows for the ubiquitination and subsequent destruction of transcription factors by the proteasome and such molecules are known as proteolysis targeting chimaeras (PROTACs) (28).

Currently we still treat AML with chemotherapy, often with treatment regimes that have not changed for decades. The experiments described above suggest that we may be at the advent of developing methods to beat cancers at the game of reprogramming transcriptional networks by the development of truly novel and potent therapies for patients.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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