Extended Background and Introduction of our study

Although the cure rate for T cell Acute Lymphoblastic Leukemia (T-ALL) has been improved dramatically during the last couple of decades, the overall prognosis remains dismal, due to frequent disease relapse and the absence of non-cytotoxic targeted therapy options. We and others have recently addressed the role of epigenetic regulation in T-ALL initiation and progression. Despite the fact that drugs targeting the function of key epigenetic factors, such as histone deacetylase (HDAC) and DNA methyltransferase (DNMT)[1,2], have been approved in the context of hematopoietic disorders, "epigenetic" drugs are currently not used for T-ALL treatment. The recent identification of mutations affecting chromatin modulators in a variety of leukemias[3-8] along with a plethora of recently generated animal models of disease have shed light on the mechanisms of action for this class of epigenetic modifiers in blood cancers. Nevertheless, there is an unmet need for development and utilization of drugs that target the epigenome[9,10-13] in pediatric acute leukemia.

To study the interplay between chromatin state and activity of chromatin modifiers in T-ALL *in vivo* we have generated and studied NOTCH1-induced disease animal models[14] (Fig. 1a). Since activating mutations in NOTCH1 are a defining feature of >50% of T-ALL cases, this model closely recapitulates many features of human T-ALL, including early developmental arrest in T-cell development, with severe blast infiltration observed in bone marrow and secondary lymphoid tissues. Using molecular and biochemical assays in this mouse model combined with functional and genetic data from primary T-ALL samples, we recently revealed a key tumor-suppressor function for the Polycomb Repressive Complex 2 (PRC2) that catalyzes methylation of lysine 27 of histone 3 (H3K27) in this type of leukemia[15]. These studies also highlighted a pivotal role for the repressive mark trimethylation of H3K27 (H3K27me3) in leukemogenesis and have demonstrated direct correlation of NOTCH1 binding and H3K27me3 loss during progression of the disease. Since net H3K27me3 levels are dictated by the balance between histone methylation and active demethylation, we hypothesized that removal of methyl groups from H3K27 is also an important process in T-ALL development. We have therefore investigated possible roles for enzymes with H3K27 demethylase activity in T-ALL.

There are currently two characterized H3K27 demethylases that belong to the Jumonji family of deoxygenases. UTX[16,17] (KDM6A) is a ubiquitously expressed protein that controls basal levels of H3K27me3, whereas JMJD3[16,17] (KDM6B) is induced upon inflammation[18], viral and oncogenic stimuli[19-21]. JMJD3 is important for neuronal differentiation[22] and promotes epidermal cell differentiation[23]. UTX, in turn, is important for induction of ectoderm and mesoderm differentiation[24,25]. Both have been shown to promote differentiation through expression of the HOX genes[26,27]. Interestingly, JMJD3 and UTX have been found to play different roles in embryonic stem cell physiology, where JMJD3 has been found to inhibit reprogramming with its dual function on INK4a/Arf expression and by mediating PHF20 ubiquitination[28], whereas UTX seems to be essential for reprogramming[29]. Despite such compelling results in developmental systems, our overall understanding of H3K27 of demethylases in cancer remains extremely limited[20,21]. UTX has been found to control cell fate[30] and to be implicated mainly in solid tumors and less in hematological malignancies[30-33], a finding supported mainly through the identification of inactivating mutations[7,33-35]. However, the roles of these two demethylases as direct modulators of the oncogenic state are largely uncharacterized.

Extended reference to *UTX* mutations

Initially, analysis of primary human samples of pediatric T-ALL using single nucleotide polymorphism (SNP) array[36] identified two patients with focal deletions of the *UTX* locus (Fig. 3f). Further targeted analyses of the same patient cohort using Sanger sequencing led to identification of three more patient cases with frameshift mutations, (Fig. 3g, Extended Data Fig. 5 and Supplementary table 2). Furthermore, we screened all coding exons of *UTX* for the presence of somatic mutations in an extended series of adult T-ALL cases (Fig. 3g and Extended Data Fig. 5). This analysis revealed the presence of *UTX* mutations in three samples analyzed. The *UTX* mutations included an in-frame deletion (p.A14_A17del), a missense (I598V) and an exon 4 splice acceptor site mutation.

Extended Discussion

Acute lymphoblastic leukemia consists of a panel of aggressive hematopoietic malignancies characterized by an array of mutations frequently affecting epigenetic modulators. Currently, there

are no targeted therapies available for treatment of this disease, leaving chemotherapy and irradiation as the only available strategies, both exhibiting severe side effects and toxicity. We propose here the targeting of JMJD3 as a novel therapy option for pediatric and adult T-ALL. This proposal is based on recent studies[3,37,38] that demonstrate that the repressive chromatin mark trimethylation of lysine 27 on histone H3 (H3K27me3) plays a key role in T-ALL, through interplay with oncogenic NOTCH1. Mechanistically, we have previously shown that the PRC2 complex antagonizes the NOTCH1 oncogenic action[37]. As NOTCH1 does not exert demethylase activity itself, we sought to delineate the exact mechanism through which NOTCH1 leads to H3K27me3 depletion from its target loci. We demonstrate here that NOTCH1-mediated JMJD3 recruitment on promoters of key T-ALL gene regulators is at least in part this mechanism (Extended Data Fig. 10).

Despite the fact that tumor suppressor roles were attributed to JMJD3 in the past[20,21,39], mainly because of the role in oncogene-induced activation of the *INK4A/ARF* (*CDKN2A*) locus followed by senescence, we have shown that this does not apply in T-ALL, where INK4A/ARF is frequently inactivated. The fact that JMJD3, in contrast to UTX, is prone to activation through specific stimuli, renders it an ideal partner and modulator of oncogenic pathways. As in the case of inflammation, T-ALL-specific *JMJD3* expression is controlled through NF-kB activation, leading to an intriguing feedback loop between NOTCH1 and NFkB pathways. Indeed, this study provides a link between these two major pathways through a specific epigenetic modulator and underlines molecular similarities between inflammation and cancer in cells of hematopoietic origin.

It is known that in the absence of NOTCH1, RBPJk interacts with co-repressor complexes (including the SMRT complex and histone deacetylases). Part of the repressive activity in these loci is filtered through the activity of PRC2 complex, which leads to high H3K27me3 levels. We propose that NOTCH1 recruitment leads to PRC2 eviction due to active demethylation of lysine 27 on histone 3 through the catalytic activity of JMJD3 and the recruitment of JMJD3 to target promoters leading to lower H3K27me3 levels. On the other hand, the reported increased levels of the activating H3K4me3 mark on a large fraction of NOTCH1 targets[15,40-42] (Fig. 4) and can be explained by the fact that NOTCH1 has the ability to recruit MLL complexes (Extended Data Fig. 2 and 10). Our findings show that both NOTCH1 and JMJD3 interact with WDR5 (Extended Data Fig. 2), providing a potential link between the fine-tuning of these two histone marks.

The GSKJ4 epigenetic inhibitor, targeting H3K27 demethylase activity, was previously tested in conditions of inflammatory stress[10]. We now show for the first time anti-tumorigenic activities and significant specificity towards NOTCH1-transformed T-ALL. We propose that GSKJ4 inhibits JMJD3 demethylase activity (see Extended Data Fig. 10), targeting T-ALL growth without affecting other cell types. Obviously, we cannot exclude the possibility that GSKJ4 could potentially affect other important epigenetic modulators or signaling pathways[43]. Nevertheless we believe that the main action of this inhibitor passes through the inhibition of JMJD3 activity as knockdown of members of other Jumonji family-potential candidates did not affect viability of T-ALL cells in sh*RNA* screens (*data not shown*). We propose that such inhibitors should be tested either as single drugs or in combination to standard chemotherapy. This notion is further supported by the fact that GSKJ4 is active at achievable concentrations in clinical settings, it is more active against JMJD3 and it affects specifically acute lymphoblastic leukemia but not healthy cells or other types of leukemia, underscoring the specificity of the compound.

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