IN THE SPOTLIGHT

Reduced PU.1 Expression Collaborates with *Tet2* Loss to Trigger Myeloid Leukemogenesis

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Summary: The leukemic transformation of hematopoietic stem and progenitor cells in the setting of *Tet2* deficiency is driven by PU.1 gene network loss through complementary reduction in PU.1 expression and hypermethylation of ETS loci at the enhancers of PU.1 target genes.

See related article by Aivalioti et al., p. 444 (6).

Lifelong blood homeostasis is maintained by a hierarchal system of hematopoietic stem and progenitor cells (HSPC) that sustain a balanced lineage output of diverse mature blood cells. However, during aging this homeostasis is jeopardized by clonally expanding HSPC that harbor somatic mutations associated with myeloid malignancy: a phenomenon known as clonal hematopoiesis of indeterminate potential (CHIP) or age-related clonal hematopoiesis (ARCH; ref. 1). Comprehensive sequencing analyses have revealed a variety of mutations associated with CHIP/ARCH; however, the most common genetic abnormalities are loss-of-function mutations in epigenetic modifiers *DNMT3A*, *TET2*, and *ASXL1* (1). CHIP/ARCH has been associated with a variety of pathologic states including diabetes, cardiovascular disease, all-cause mortality, and leukemia (1).

The mechanism(s) underlying selective expansion of mutant HSPC in the setting of CHIP/ARCH and their evolution to malignancy remain poorly understood. Interestingly, previous literature suggests reduction of PU.1 expression and/or function triggered by a variety of leukemia-associated mutations could serve as a common mechanistic factor underlying progression to malignancy (2, 3). PU.1 is an ETS-family transcription factor that serves as a master regulator of hematopoietic stem cell lineage output. Specifically, PU.1 regulates genes that establish an appropriate balance between myeloid lineage specification, cell-cycle activity, and hematopoietic stem cell (HSC) self-renewal (4, 5). Previous work has shown that reduced PU.1 expression or activity can establish a preleukemic state that cooperates with DNA repair deficiency to promote leukemic transformation and acute myeloid leukemia (AML)–like disease in mice (2). As mutations associated with CHIP/ARCH are common first stops for HSPC on the road to malignant transformation, the extent to which they interact with graded reductions in PU.1 activity associated with myeloid malignancy remains poorly understood. In this issue of *Blood Cancer Discovery*, Aivalioti and colleagues further our insight into the process of malignant transformation by exploring how the combinatorial loss of Tet2 and PU.1 triggers myeloid leukemogenesis (6).

To establish whether loss of PU.1 activity contributes to malignant transformation in the setting of *TET2* deficiency, Aivalioti and colleagues used a murine system by which they could determine whether compound reductions in PU.1 activity and *Tet2* expression facilitate AML development. These researchers had previously shown that reductions of PU.1 through heterozygous deletion of the 14-kb upstream regulatory element (URE) of the PU.1-encoding *Spi1* gene result in a 35% reduction of PU.1 expression that, when combined with impairment of DNA mismatch-repair components MutSα and MutSβ, results in AML-like disease (2). As loss of *Tet2* also drives aberrant self-renewal without inducing leukemic transformation in HSPC (7), Aivalioti and colleagues generated a conditional compound mutant murine model that harbors heterozygous or homozygous deletion of the *Spi1* URE ($PU.1^{URE\Delta/+}$) in combination with either heterozygous or homozygous *Tet2* deletion ($Tet2^{+/flox}$ and $Tet2^{flox/flox}$ on a *Vav-Cre* background) to investigate whether reduced PU.1 levels cooperate with Tet2 deficiency to trigger leukemogenesis. Notably, neither $PU.1^{URE\Delta/+}$::$Tet2^{+/flox}$ nor $PU.1^{URE\Delta/+}$::$Tet2^{flox/flox}$ (hereafter referred to as $URE^{HET}Tet2^{HET}$ and $URE^{HET}Tet2^{KO}$, respectively) mutant mice showed hematologic perturbations at a young age (3–5 months). However, aged compound mutant mice showed a *Tet2* dose–dependent reduction in survival, and moribund compound mutant mice showed elevated white blood cell counts and harbored increased numbers of blast cells in peripheral blood, bone marrow, and spleen, which are characteristic of myeloid malignancy. Furthermore, bone marrow cells from compound mutant mice showed aberrant clonogenic and serial replating capacity *in vitro* as well as increased leukemia-initiating potential via transplantation *in vivo*. Thus, graded reductions in PU.1 activity can cooperate with *Tet2* loss of function to trigger a highly penetrant AML phenotype, specifically in the setting of aging.

Interestingly, these researchers noted that blast cells from compound mutant mice had acquired additional cancer-related mutations in genes including *Cux1* and *Kmt2d* among

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others, indicative of intraclonal heterogeneity and progressive acquisition of additional mutations, reminiscent of the genotypic heterogeneity in human AML. Leukemic blasts from leukemic compound mutant mice exhibited a wide range of morphologic phenotypes varying between immature HSPC-like to metamyelocytic, again mirroring the heterogeneous presentation of human AML. To address the molecular phenotype and heterogeneity of leukemias in the compound mutant mice, the authors profiled their transcriptional states using RNA sequencing (RNA-seq). Strikingly, principal component analysis of these profiles revealed that HSPC from mice with a mature AML phenotype more closely resembled normal HSPC, than HSPC from mice with an immature AML phenotype. Furthermore, although PU.1 expression levels were heterogeneous among leukemic cells, they correlated more strongly with differentiation state than with malignant transformation per se.

To better understand whether the dissidence between PU.1 mRNA and protein abundance is indicative of an aberrant epigenetic mechanism established by *Tet2* loss of function, the authors first looked at differential gene expression in HSPC from compound mutant mice with AML. Notably, a majority of these DEGs harbored a conserved PU.1 binding motif, which, when overlaid with published PU.1 and Tet2 chromatin immunoprecipitation data, revealed that roughly a third of these DEGs were direct targets of PU.1 and Tet2, which is known to interact directly with PU.1. Network analysis further showed PU.1-related gene networks were compromised in both $URE^{HET}Tet2^{HET}$ and $URE^{HET}Tet2^{KO}$ mice, and subsequent pathway analysis revealed perturbations consistent with the previously described functional and morphologic phenotypes of compound mutant AML blasts. Thus, the authors' data support a model in which disruption of the PU.1 network, rather than simply a reduction in levels of PU.1 itself, underlies malignant transformation.

As the authors noted, the morphological heterogeneity in this model is reminiscent of human AML, but the paradoxical relationship between PU.1 protein abundance vs. mRNA levels in the leukemic HSPC is a potentially important focus for further investigation. There are numerous potential explanations for this discordance. On a technical level, leukemia-initiating cells can be a relatively minor population within the c-Kit$^+$ HSPC fraction, and hence reductions in PU.1 protein may not be easily read out in this heterogenous compartment. In this context, the dysregulated PU.1 network may be established in a more primitive HSPC population (even in HSC themselves) where PU.1 mRNA and protein levels are concordantly reduced. Impaired PU.1 network activity could thus be durably transmitted to leukemic progeny cells that otherwise possess a relatively mature myeloid identity and express high levels of PU.1 protein (8). Lastly, while the leukemic c-Kit$^+$ HSPC express abundant PU.1 protein, deactivating post-translational modifications or even leukemia-associated changes in stochastic mechanisms regulating transcription factor dynamics, could nonetheless impair PU.1 function without reducing its overall expression. Further studies that address the relationship between PU.1 gene dosage, protein levels and leukemic transformation in different HSPC populations, and the extent to which impaired PU.1 network activity is an inherited feature passed on from primitive cells like HSC to more lineage-committed progeny, will clarify this paradox.

To address the epigenetic component of their model, the authors performed transposase-accessible chromatin by sequencing (ATAC-seq) of leukemic HSPC relative to normal HSPC and overlapped the observed changes in chromatin states with *cis*-regulatory elements (cCRE) from a published murine transcriptome database. This comprehensive analysis revealed reductions in DNA accessibility at monocyte and neutrophil-specific enhancer cCREs and promoters. By clustering alterations of enhancer-originating RNA transcripts (eRNA) with differential chromatin peaks, the authors also found that HSPC from compound mutant mice with AML share a regulatory pattern with myeloid progenitors. Moreover, cCREs of compound mutant leukemic HSPC that harbor either PU.1 or Tet2 targets were preferentially enriched in regions of closed chromatin. To determine whether this model of PU.1 network disruption is a common molecular theme in leukemic HSPC, the authors compared their DEG data with published AML and myelodysplastic syndrome (MDS) patient data sets. They found striking similarities between the two data sets, with common reductions in PU.1 network genes. Similar to the authors' own RNA-seq analyses, reduced PU.1 network activity in human MDS and AML was independent of PU.1 mRNA levels. However, the authors found the DEGs from their leukemic HSPC were enriched for genes repressed by an oncogenic fusion gene, *ETV6-RUNX1*. This fusion gene results in a repressor protein that bears the N-terminus of *ETV6* (an ETS family member that allows recruitment of corepressors and histone deacetylases) and the DNA-binding domain of RUNX1 (a hematopoietic master regulator that itself activates expression of *Sfpi1*). Although compound mutant mice did not have the *ETV6–RUNX1* fusion gene itself, gene expression and chromatin accessibility at *ETV6-RUNX1* signature genes were reduced in compound mutant HSPC relative to normal HSPC. Furthermore, the majority of cCREs of *ETV6–RUNX1*-signature genes possessed PU.1-binding sites, consistent with the role of this oncogenic fusion in repressing ETS family transcription factors. Interestingly, HOMER motif analysis of the cCRE sequences with PU.1-binding sites in compound mutant HSPCs identified a core PU-box motif with flanking cytosines that would leave them more susceptible to methylation. Thus, the presence of these methylation-sensitive ETS loci (referred to as *methETS*) may impede PU.1 activity in settings where DNA methylation is perturbed, such as in the context of *TET2* mutation. Indeed, using bisulfite sequencing, the authors show that leukemic HSPC exhibit DNA hypermethylation at enhancers containing PU.1 *methETS* sequences, leading to subsequent reduction in gene expression relative to age-matched nonleukemic HSPC. Thus, the presence of these hypermethylated *methETS* sites appears to constitute a mechanism by which Tet2-mediated enhancer hypermethylation synergizes with minimal loss of PU.1 expression to drive dysregulation of the PU.1 network and subsequent leukemic transformation during aging (Fig. 1), which complements and extends previous work establishing a role for PU.1 deficiency in leukemogenesis (2, 3, 6).

This work raises several important questions and next steps for the field. Mutations in *TET2* are often, though not exclusively, associated with early stages of leukemogenesis. Thus, one can speculate that hypermethylation of *methETS* loci can

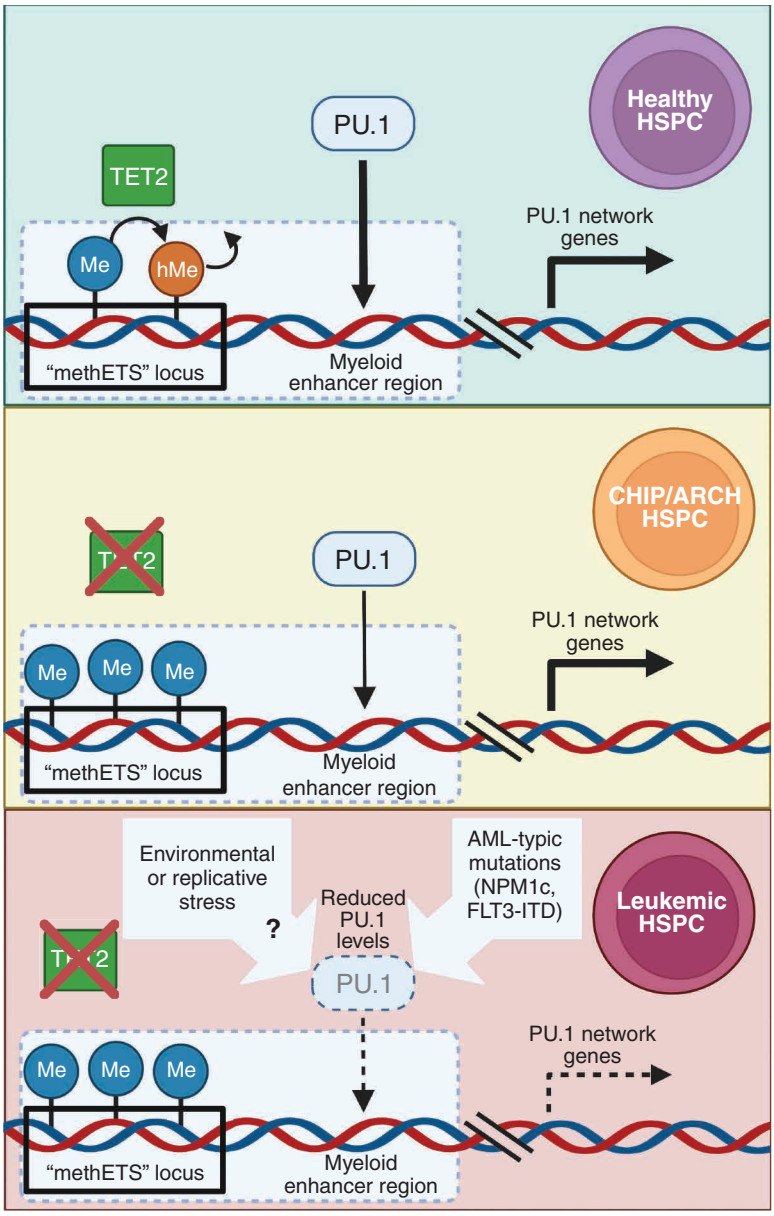

Figure 1. In healthy hematopoietic stem and progenitor cells (HSPC), TET2 promotes the first step in cytosine demethlation, the conversion of methylcytosine (Me; blue lollipop icons) to hydroxymethylcytosine (hMe; orange lollipop icons) residues at the enhancer regions of myeloid target genes. This allows appropriate PU.1 binding and subsequent transactivation of myeloid maturation genes. In the context of TET2 deficiency associated with clonal hematopoiesis of indeterminate potential/age-related clonal hematopoiesis (CHIP/ARCH) loss of TET2 activity in HSPC results in hypermethylation of specific ETS binding sites located in many PU.1 target gene enhancers (termed by the authors as methylation-sensitive ETS, or methETS, loci; depicted by black box outline) which in turn may reduce the capacity for PU.1 to transactivate gene expression at these targets (depicted by reduced thickenss of the PU.1 arrow). AML-typic mutations and potentially other aging-related environmental factors that reduce PU.1 expression can act in concert with hypermethylation of methETS loci to further abolish PU.1 binding at these regions (depicted by broken lines around PU.1 and arrow). Resultant dysregulation of the PU.1 network ultimately results in leukemic transformation of HSPC.

establish a cellular context that is permissive for leukemic transformation following acquisition of subsequent AML-typic mutations that reduce PU.1 levels. The combinatorial genetic knockouts used by Aivalioti are present from fetal life onward and thus the system does not directly address how *TET2* loss and reduced PU.1 levels may act sequentially to drive leukemogenesis, including whether the process can work in reverse, with *TET2* loss triggering leukemogenesis in the PU.1-deficient setting. The degree to which order of acquisition and/or combinations of specific mutations progressively deregulate the PU.1 network—and the extent to which therapeutic interventions can restore its function—remain open questions with important clinical implications. Novel mouse systems under development that use inducible mutant alleles to replicate sequential acquisition of AML-associated mutations and even allow for their reversal are likely to prove useful tools for addressing this question.

The authors' combinatorial mutant model provides a compelling testbed for understanding the broader somatic evolutionary and molecular contexts of AML pathogenesis. It is noteworthy that the combinatorial model used by Aivalioti and colleagues only exhibits leukemic transformation upon aging, clearly matching the pattern of AML incidence in humans. Furthermore, the onset and penetrance of the leukemia is variable, particularly in $URE^{HET}Tet2^{HET}$ mice, where mutant gene dosage is lowest but also most reflective of likely mutant allele burden in humans. These findings suggest factor(s) aside from the presence of the lesions themselves influence leukemia evolution. Further studies can address the relative contributions of subclonal patterns of evolution in compound mutant HSPC (particularly the authors' finding that leukemic HSPC had sometimes acquired subsequent mutations in genes like *Cux1*), the overlay of aging-associated epigenetic deregulations, as well as environmentally driven

mechanisms of competition between normal and mutant clones. Indeed, we recently showed that inflammation driven by interleukin-1 can trigger the expansion of PU.1-deficient HSC, as these cells fail to induce PU.1-dependent molecular mechanisms that suppress protein synthesis and cell-cycle activity (4). Of note, many of the downregulated PU.1 network genes described in this study are associated with inflammatory pathways, suggesting the leukemic HSPC may be refractory to these signals *in vivo*, thus facilitating their selection in the aged setting. Using models such as this as a test bed for identifying novel metabolic, anti-inflammatory, and/or epigenetic strategies that target the downstream effects of PU.1 network deregulation could improve the potential for LSC eradication and/or prevention of disease progression.

The extent to which this mechanism is generalizable to mutations in other epigenetic regulators and even other CHIP/ARCH-associated mutations more broadly, i.e., *DNMT3A*, *ASXL1*, splice factors, etc., is an exciting area into which these studies can be extended. Recent work characterizing the methylomes of PBMCs from individuals with *TET2* and *DNMT3A* CHIP/ARCH identified aberrant hypermethylation of PU.1 targets in *TET2*-mutant cells (thus nicely complementing this study), whereas *DNMT3A*-mutant cells displayed a unique pattern of epigenetic dysregulation (9). One might anticipate that mutations that phenocopy features of *TET2* loss (such as mutations in IDH2) may be most likely to disrupt the PU.1 network via a similar mechanism. Furthermore, the degree to which order of acquisition and/or combinations of specific mutations progressively deregulate the PU.1 network—and the extent to which therapeutic interventions can restore its function—remains an open question with clinical implications. Novel mouse systems that replicate sequential acquisition of AML-associated mutations and even allow for their reversal are likely to prove useful tools for addressing these questions. To this end, novel CRISPR-based tools that facilitate similar studies of compound mutations in human HSPC will prove useful in both basic science and preclinical modeling scenarios. As an example, Dr. Ravindra Majeti's group uses a CRISPR/Cas9-mediated approach to model *TET2* loss in primary human HSPC (10), demonstrating the power of this system to study key functional and epigenetic dysregulations associated with CHIP/ARCH in humanized mouse systems. As they report cell-type–specific methylation landscapes that affect chromatin accessibility using this model, there is an opportunity for translating the work described by Aivalioti and colleagues to a human setting.

Altogether, the authors' work provides valuable molecular and mechanistic insight into the cooperation between epigenetic dysregulation and reduced PU.1 levels associated with AML-typic mutations. These studies can be used as a basis for further investigations into the cooperativity between mutations in epigenetic regulators and PU.1 network dysregulation in myeloid malignancy pathogenesis, as well as the extent to which this process can be effectively targeted by novel and existing therapies.

Authors' Disclosures

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