

C/EBP α : critical at the origin of leukemic transformation

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Acute myeloid leukemia (AML) is a hematopoietic malignancy characterized by clonal expansion of myeloid progenitor cells. A major mechanistic theme in AML biology is the extensive collaboration among fusion oncoproteins, transcription factors, and chromatin regulators to initiate and sustain a transformed cellular state. A new study in this issue describes how the C/EBP α transcription factor is crucial for the initiation of AML induced by MLL fusion oncoproteins, but is entirely dispensable for the maintenance of established disease. These observations provide a unique glimpse into the pioneer round of regulatory events that are critical at the origin of AML formation. Furthermore, this study implies the existence of oncogene-induced positive feedback loops capable of bypassing the continuous need for certain regulators to propagate disease.

Acute myeloid leukemia (AML) is a blood cancer characterized by uncontrolled proliferation of myeloid progenitors that exhibit a severe block in their ability to differentiate into mature granulocytes or macrophages. A critical event in AML initiation is the acquisition of aberrant self-renewal characteristics in leukemia stem cells (LSCs), a cell population that has been associated with disease propagation and the evolution of therapy-resistance. The estimated 5 yr survival rate among AML patients is \sim 30%, with mortality being largely attributed to the emergence of chemotherapy resistance. Deregulated transcriptional pathways feature prominently in the genetic etiology of AML, which can occur in the form of gain- or loss-of-function mutations in lineage-specific transcription factors, histone modifying enzymes, and DNA methylation machineries (Rosenbauer and Tenen, 2007; Shih et al., 2012). Of clinical relevance, pharmacological targeting of transcriptional regulatory proteins represents a validated approach for undermining the driver oncogenes in certain subtypes of AML, as exemplified by the clinical success of using all-trans retinoic acid and arsenic trioxide to inactivate fusion oncoproteins involving the

retinoic acid receptor (Wang and Chen, 2008; Ablain et al., 2013).

One important genetic subtype of AML is defined by rearrangements of the *mixed lineage leukemia (MLL)* gene, which is found in \sim 5% of patients (Krivtsov and Armstrong, 2007). *MLL* encodes a histone-modifying enzyme belonging to the SET1 family of H3 lysine 4 (H3K4) methyltransferases, which often function as transcriptional coactivators (Shilatifard, 2012). In AML, chromosomal translocations can involve *MLL* and various other genes, of which $>$ 50 partner loci have been described to date (Krivtsov and Armstrong, 2007). Some of the most common translocation partners of *MLL* include *AF9*, *ENL*, *AF4*, *AF6*, *AF10*, and *ELL*. The in-frame fusion of the N terminus of *MLL* with the C terminus of its fusion partner leads to the formation of a potent oncoprotein (e.g., *MLL-ENL*) that can efficiently transform hematopoietic stem and progenitor cells. This genetic driver that has been widely used to generate animal models of leukemia for studying all aspects of disease biology, including LSC specification and chemotherapy resistance (Krivtsov et al., 2006; Somervaille and Cleary, 2006; Zuber et al., 2009). *MLL*-rearranged leukemia is considered a poor/intermediate prognosis subtype of leukemia for which targeted therapies have yet to make any clinical impact (Krivtsov and Armstrong, 2007).

Mechanistically, *MLL* fusion proteins form hybrid multisubunit protein complexes that include factors associated with the *MLL* N-terminal fragment (Menin, LEDGF, and PAF1) and factors associated with the fusion partner C-terminal fragment (Mohan et al., 2010). Remarkably, many of the common C-terminal fusion partners associate with one another in a complex that includes the protein kinase pTEFb and the histone methyltransferase DOT1L, which act as effectors to promote transcription elongation (Okada et al., 2005; Lin et al., 2010; Yokoyama et al., 2010). Because *MLL* fusion proteins lack the domains required for catalysis of H3K4 methylation, they instead rely on the C-terminal fusion partner to recruit pTEFb and DOT1L to promote transcriptional activation of their target genes (Okada et al., 2005). Among the well-validated direct targets of *MLL* fusion proteins are genes encoding homeodomain-containing transcription factors, including *Hoxa9* and *Meis1* (Armstrong et al., 2002). Forced co-overexpression of *Hoxa9* and *Meis1* can itself efficiently transform hematopoietic cells and form leukemias that resemble those initiated by *MLL* fusion proteins (Kroon et al., 1998). In addition to *Hoxa9/Meis1*, *MLL* fusion proteins also activate the expression of numerous additional transcription factor-encoding genes, such as *Myc*, *Myb*, and *Mef2c*, which are crucial for disease maintenance (Krivtsov et al., 2006; Zuber et al., 2011a). Furthermore, *MLL* fusion leukemia cells are also dependent on chromatin regulatory activities to maintain a transformed cellular state, such as the histone demethylase LSD1 and the BET protein BRD4 (Zuber et al., 2011b; Harris et al., 2012). Thus, *MLL*-rearranged

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leukemia is a paradigm for understanding how a vast network of transcriptional regulators cooperates to sustain an aberrant lineage program. A new study in this issue of the *JEM*, Ohlsson et al., demonstrates an essential collaboration between MLL fusion proteins and the transcription factor C/EBP α during leukemogenesis. Interestingly, C/EBP α is critical for the initiation of MLL fusion leukemia but becomes dispensable once the disease is fully established.

Function of C/EBP α in normal and malignant myelopoiesis

The CCAAT/enhancer-binding protein- α (C/EBP α) is a lineage-specific transcription factor in the hematopoietic system that is required for the formation of committed myeloid progenitors from multipotent precursor cells (Rosenbauer and Tenen, 2007). C/EBP α executes this function by coupling the direct transcriptional activation of myeloid-specific genes with the arrest of cell proliferation (Nerlov, 2004). C/EBP α belongs to a subfamily of basic-region leucine zipper (BR-LZ) transcription factors, several of which are also expressed in the myeloid lineage (e.g., C/EBP β and C/EBP ϵ ; Rosenbauer and Tenen, 2007). C/EBP α is an intronless gene whose mRNA can be translated from two different AUG codons to give rise to two distinct isoforms (p42 and p30). p30 lacks two N-terminal trans-activation domains that are only present on p42. Notably, this unique N-terminal region of p42 can interact with and inhibit E2F transcription factors (Slomiany et al., 2000; Porse et al., 2001). Hence, only the p42 isoform of C/EBP α can promote proliferation arrest. p30 retains the BR-LZ and a third trans-activation domain and can promote myeloid lineage specification, but lacks the capacity to promote terminal differentiation and proliferation-arrest (Kirstetter et al., 2008). The relative levels of p42 and p30 in the cell can be regulated at the level of translation initiation through mTOR signaling to control cell fate transitions (Calkhoven et al., 2000). Notably, a genetic knockout C/EBP α (p30 and p42) results in a complete block in the transition from common myeloid progenitor (CMP) to the granulocyte/monocyte

progenitor (GMP) stage of differentiation and leads to enhanced self-renewal of hematopoietic stem cells, highlighting the essential function of C/EBP α for normal hematopoiesis (Zhang et al., 2004).

Genetic alteration of C/EBP α is a major etiology of human AML, with a mutational frequency \sim 10% across this disease (Pabst et al., 2001b). C/EBP α mutations fall into two major categories: those that abolish p42 protein expression while retaining p30 and insertions/deletions near the BR-LZ region that compromise DNA binding, which may result in dominant-negative homodimers or heterodimers with other C/EBP family members (Nerlov, 2004). Interestingly, these two classes of mutations often co-occur in a biallelic manner in AML patients. C/EBP α expression can also be down-regulated through direct transcriptional repression by fusion oncoproteins, such as AML1-ETO (Pabst et al., 2001a). A selective knockout of p42 in mice (while preserving p30 expression) leads to AML formation with complete penetrance, confirming the p42 isoform of C/EBP α as a myeloid tumor suppressor gene (Kirstetter et al., 2008). Null mutations of C/EBP α that abolish both p42 and p30 are not observed in human AML, and mice with a complete knockout of p42/p30 fail to develop AML, suggesting that residual C/EBP α function (presumably via the p30 isoform) is necessary to form myeloid lineage leukemias (Zhang et al., 2004). Consistent with this possibility, C/EBP α knockout bone marrow will not form myeloid leukemias when transduced with BCR-ABL, but instead will only form leukemias of the erythroid lineage in this genetic background (Wagner et al., 2006). These results highlight a critical dosage-dependent role of C/EBP α in AML pathogenesis: partial loss of function (e.g., through selective loss of p42) can drive AML progression whereas complete loss (e.g., through complete loss of p30/42) might be incompatible with leukemias of the myeloid lineage.

C/EBP α is essential for the initiation but not the maintenance of MLL fusion leukemia

In an effort to discover novel transcription factors that support MLL fusion-mediated

leukemogenesis, Ohlsson et al. (2013) noted that C/EBP α target genes tended to be more highly expressed in MLL-rearranged human AML samples when compared with normal hematopoietic progenitors. To determine the functional relevance of this observation, the authors evaluated whether C/EBP α knockout hematopoietic stem and progenitor cells were capable of being transformed by the MLL-ENL oncogene. Importantly, in this experimental design they first inactivated C/EBP α in vivo with conditional alleles, followed by retroviral transduction of MLL-ENL into the hematopoietic stem and progenitor cells isolated from these animals. The authors found that C/EBP α was absolutely essential for MLL-ENL-mediated transformation in colony formation assays, liquid culture, and after transplantation in vivo. Based on prior studies (such as the aforementioned BCR-ABL study), this result was not entirely surprising. C/EBP α is critical in the formation of normal GMPs, and thus a failure to form myeloid lineage leukemias may simply reflect the normal lineage requirement for C/EBP α in myeloid progenitors. To address this, the authors first sorted GMPs from C/EBP α ^{flxed/flxed} mice and subsequently inactivated C/EBP α , since upon reaching the GMP stage C/EBP α has been shown to be dispensable for further myeloid maturation (Zhang et al., 2004). Importantly, C/EBP α -null GMP cells were still unable to be transformed by MLL-ENL, suggesting an intrinsic requirement for C/EBP α that is independent of lineage establishment (Ohlsson et al., 2013). C/EBP α -knockout cells also failed to be transformed by co-transduction with Hoxa9/Meis1, indicating that a defect in MLL-ENL-mediated activation of these two downstream target genes was not the primary cause of impaired leukemogenesis. This finding also suggests that the C/EBP α requirement is not restricted to MLL fusion AML, but applies to other oncogenes that activate related pathways. However, C/EBP α is not required for transformation induced by the E2A-HLF oncogene, demonstrating that C/EBP α is only required for leukemogenesis in specific genetic contexts.

Extending these observations to the setting of disease maintenance, Ohlsson et al. (2013) went on to evaluate whether conditional inactivation of C/EBP α after MLL-ENL transduction also impaired cell expansion. Remarkably, conditional inactivation of C/EBP α in MLL-ENL-transformed cells had no effect on colony formation in serial replating assays or under in vivo conditions. C/EBP α -null AML could also be readily transplanted into tertiary recipients without any delay in disease progression, highlighting the dispensability of C/EBP α in leukemia stem cell subpopulations. These findings provide a dramatic example where a gene can be required exclusively at the stage of cancer initiation but is rendered superfluous in the setting of a fully transformed state.

What could be the mechanistic basis for the C/EBP α -requirement for leukemia initiation? Ohlsson et al. (2013) found that the majority of genes induced immediately after MLL-ENL transduction were not properly induced in C/EBP α -null cells. Furthermore, CHIP-seq analysis revealed that C/EBP α binding to DNA correlates with MLL-ENL target genes. These observations suggest, although do not prove, that C/EBP α binding to DNA might be a prerequisite for MLL fusion proteins to efficiently occupy chromatin during the initial stages of leukemogenesis. Such a model, however, cannot explain why C/EBP α is also required for initiation of AML by Hoxa9/Meis1 overexpression, since these cells lack an MLL fusion protein. It is interesting to note that a prior CHIP-seq analysis performed in leukemia cells found a close correlation between C/EBP α and Hoxa9/Meis1-binding sites at a large number of distal enhancer elements (Huang et al., 2012). This observation raises the interesting possibility that C/EBP α might also prime chromatin for subsequent binding of Hoxa9/Meis1 proteins at regulatory elements. Such a function would be reminiscent of pioneer transcription factors, described previously as a class of DNA-binding proteins that render chromatin competent for other factors to bind (Zaret and Carroll, 2011).

Open questions

Perhaps the most provocative question raised by this study is why C/EBP α becomes dispensable after full leukemic transformation. Although this issue was not directly addressed in this study, one can envision several regulatory scenarios that could explain this result. MLL fusion proteins are known to induce the expression of numerous additional transcription factors (e.g., Myb, Mef2c, Myc; Zuber et al., 2011a). These transcription factors, once achieving high levels of expression in transformed cells, might themselves be capable of stabilizing MLL fusion protein recruitment to chromatin, thereby establishing an oncogene-induced

positive feedback loop (Fig. 1). Myb has previously been shown to physically associate with MLL fusion proteins and would be a logical candidate for bypassing the C/EBP α requirement for oncoprotein recruitment to DNA (Jin et al., 2010). Other C/EBP family homologues (e.g., C/EBP β) expressed in fully transformed MLL fusion leukemia cells would be additional candidates that could alleviate the C/EBP α requirement after leukemic transformation. An alternative explanation for a transient C/EBP α requirement could be that MLL fusion proteins regulate distinct genes at different temporal stages of leukemic transformation. In this situation, C/EBP α is

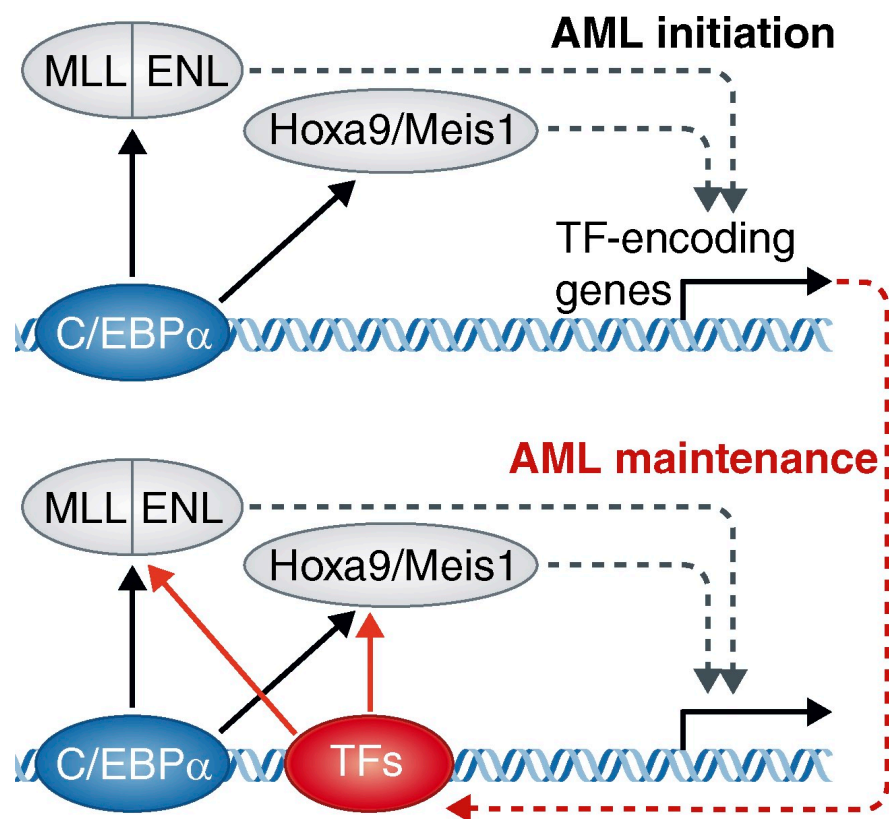


Figure 1. A speculative model to account for a transient C/EBP α requirement during initiation of MLL-ENL- or Hoxa9/Meis1-induced acute myeloid leukemia. (top) At the onset of oncoprotein expression, C/EBP α is critical for initial recruitment of MLL-ENL or Hoxa9/Meis1 to their target sites on DNA, as depicted by arrows pointing from C/EBP α to these factors. Such an effect could be mediated through direct physical interactions or indirectly by increasing DNA accessibility. Many of the known target genes of MLL-ENL and Hoxa9/Meis1 are other transcription factors (TFs), hence C/EBP α would be critical to drive the initial burst in expression of these factors. (bottom) Upon reaching a fully transformed state, these additional TFs (encoded by genes up-regulated by the oncoproteins) would now have achieved sufficient levels of expression to participate in stabilizing oncoprotein recruitment. In this situation, they might act in parallel with (or replace) C/EBP α at MLL-ENL- or Hoxa9/Meis1-binding sites. In this fully transformed state, the C/EBP α requirement would be alleviated by these alternative TFs.

critical for the primary wave of regulatory events that occur upon leukemia initiation, but becomes dispensable as disease progresses; as MLL-ENL occupancy on chromatin shifts to a secondary set of target genes that are regulated in a C/EBP α -independent manner. Elucidating the mechanistic basis for the transient role of C/EBP α during AML progression will be a worthwhile endeavor and may redefine our understanding of the stepwise progression of leukemogenesis.

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