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C/EBPα deregulation as a paradigm for leukemogenesis

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Abstract

Myeloid master regulator CCAAT enhancer-binding protein alpha (C/EBPα) is deregulated by multiple mechanisms in leukemia. Inhibition of C/EBPα function plays pivotal roles in leukemogenesis. While much is known about how C/EBPα orchestrates granulopoiesis, our understanding of molecular transformation events, the role(s) of cooperating mutations and clonal evolution during C/EBPα deregulation in leukemia remains elusive. In this review, we will summarize the latest research addressing these topics with special emphasis on *CEBPA* mutations. We conclude by describing emerging therapeutic strategies to restore C/EBPα function.

INTRODUCTION

Hematopoiesis is the process by which multipotent hematopoietic stem cells (HSCs) develop into mature blood cells through a highly organized hierarchy of successive differentiation events. Transcription factors play a central role in the multi-layered regulation of hematopoiesis. Perturbations in transcription factor activity result in a differentiation block, lineage infidelity and transformation events, which lead to leukemia.[1]

The C/EBP family of transcription factors consists of six members—C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPζ and C/EBPε.[2] C/EBPα, the founding member of C/EBP family, was initially characterized in adipogenesis and later found to be expressed in multiple tissues, including the liver, lung, skin, mammary glands and hematopoietic cells, acting as a tumor suppressor. Studies on C/EBPs led to the discovery of the basic region leucine zipper (bZIP) and the basic helix-loop-helix (bHLH) classes of transcription factors.[3,4] Research on C/EBPα has contributed immensely to our understanding of transcription factor function in

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CONFLICT OF INTEREST

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lineage specificity, cell cycle control by transcription factors, the transcriptional program of differentiation, and the molecular basis for transformation in cancer.[1,5]

C/EBPα FUNCTION IN HEMATOPOIETIC DIFFERENTIATION

Development of HSC to various lineages is the result of two fundamental processes: self-renewal and differentiation. Both these processes are orchestrated by transcription factors by complex mechanisms.[1] The intron-less *CEBPA* gene encodes a mRNA that can translate into two major forms—42 kD C/EBPα-p42 and 30 kD C/EBPα-p30 based on alternate translation initiation codons.[6] Cells regulate the p42/p30 ratio at translation initiation by mTOR signaling and C/EBPα-p30 dominantly inhibits the C/EBPα-p42 function when the ratio of p42/p30 is less than one.[7] C/EBPα-p30 lacks two N-terminal transactivation domains that are unique to C/EBPα-p42, which play a central role in the inhibition of E2F transcription factors.[8,9]

Conditional deletion of *Cebpa* in mouse displays lack of granulocytes with a specific block in the common myeloid progenitor (CMP) to granulocyte-monocyte progenitor (GMP) step, underlining the central role of C/EBPα in granulopoiesis.[10] C/EBPα regulates myeloid differentiation by upregulating myeloid-specific genes and by blocking myeloid cell proliferation. Well characterized examples of genes regulated by C/EBPα in granulopoiesis include transcription factors (PU.1,[11] c-Jun,[12] c-Myc,[13] SOX4[(ref 14)] and E2F[9]), growth factor receptors (granulocyte colony-stimulating factor and granulocyte macrophage colony-stimulating factor), primary granule proteins (myeloperoxidase), secondary granule proteins (lactoferin)[15] and microRNAs (miR-223,[16,17] miR-34a[18] and miR-30c[19]). Even though C/EBPα is widely considered as a master regulator of granulopoiesis, reports suggest it is important in monocyte/macrophage differentiation,[20,21] suggesting the role of C/EBPα in lineage commitment is context dependent.

Even though C/EBPα is expressed at low levels in HSC, it negatively regulates HSC self-renewal.[10] Ye *et al.*[22] reported that C/EBPα controls maintenance of adult HSC quiescence, but not the survival of HSCs. Loss of C/EBPα establishes a fetal transcriptional program in adult HSCs and enhances HSC proliferation by de-repressing N-Myc.[22] The function of C/EBPα in HSCs was re-evaluated recently by Hasemann and colleagues.[23] Their study demonstrated that C/EBPα positively regulates HSC self-renewal, protects adult HSCs from apoptosis and maintains their quiescent state.[23] These studies demonstrated opposite views for the role of C/EBPα in HSC function. In these studies, the timing of C/EBPα excision could have resulted in disparate findings. In most of the experiments in Ye's study,[22] donor cells were transplanted into a recipient mouse followed by pIpC-mediated deletion. Meanwhile, in experiments that evaluated HSC function in Hasemann's study,[23] donor cells had undergone pIpC-mediated deletion followed by transplantation. So, loss of C/EBPα (before transplantation) may have compromised HSC engraftment during transplantation, resulting in lower reconstitution, which was interpreted as a competitive disadvantage in the Hasemann's study.[23] This also explains why Cebpa $^{/}$ HSCs in that study do not exhibit a proliferative advantage and eventually underwent apoptosis. In support of this possibility, C/EBPα directly regulates CXC chemokine receptor 4 (*CXCR4*), one of the major regulators

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for homing, engraftment, and quiescence of HSCs in the bone marrow niche,[24,25] and is downregulated in AML samples with *CEBPA* mutations.[26]

The discrepancies in C/EBPα-mediated HSC function are similar to previous studies, which investigated RUNX1 function in HSCs. While Growny *et al.*[27] reported that RUNX1-deficient HSCs display impaired chimerism in competitive transplantation assays, they did not observe defective LT-HSC activity; Ichikawa *et al.*,[28,29] showed that in their experimental settings RUNX1 deletion does not impair chimerism in competitive transplantation assays and is inversely correlated with LT-HSC activity. Collectively, the timing of C/EBPα excision and whether it affected engraftment of bone marrow cells in transplantation experiments could have resulted in varying interpretations of the role of C/EBPα in HSCs. Another explanation for the different views of C/EBPα function in HSCs is the timing of analysis (5–7 days after pIpC in most of the experiments in Ye's study versus ⩾ 16 weeks after pIpC in Hasemann's study).

The above-mentioned studies bring our attention to the unexplored role of C/EBPα in HSCs, which is compromised in leukemia (discussed below).

C/EBPα AND CELL CYCLE CONTROL

In addition to acting as a classical transcription factor, C/EBPα blocks cell cycle progression[30] with mechanisms that vary from cell type to cell type (reviewed in Nerlov *et al.*[31]). In hematopoiesis, inhibition of E2F transcription factors have been widely accepted as the mechanism for C/EBPα-mediated growth arrest.[9,32,33] We have showed that C/EBPα inhibits E2F by directly regulating two microRNAs miR-223 and miR-34a, which target E2F1 and E2F3 respectively.[17,18] In line with these findings, acute myeloid leukemia (AML) with C/EBPα deregulation is associated with decreased activity of miR-223 and miR-34a. Mouse models of *Cebpa* mutants suggests loss of cell cycle regulation by C/EBPα plays a major role in leukemogenesis.[34,35]

C/EBPα DEREGULATION IN AML EXCLUDING MUTATION

The fundamental characteristics of leukemia are block of differentiation in a particular lineage, leading to enhanced proliferation and cell survival resulting from inactivation of tumor suppressors and/or activation of oncogenes. The first report of C/EBPα deregulation was shown by RUNX1/ETO, the product of the t(8;21) translocation.[36] RUNX1/ETO blocks C/EBPα expression by inhibiting positive autoregulation of the *CEBPA* promotor[37] and by directly binding to the +42 kb enhancer,[38] which is regulated by RUNX1 in granulopoiesis.[39,40] CBFβ-SMMHC fusion protein found in AML patients with inv(16) inhibits C/EBPα by upregulating calreticulin, which binds to GC-rich regions in C/EBPα mRNA.[41] Treatment of inv(16) cells with AI-10-49, a CBFβ-SMMHC inhibitor can reactivate C/EBPα expression in inv(16) cells.[42] Inhibition of C/EBPα by calreticulin is also found in RUNX1-MDS-EVI1 translocation t(3;21).[43]

BCR-ABL, the product of the t(9;22) translocation in chronic myeloid leukemia upregulates poly(rC)-binding protein hnRNP-E2, inhibiting C/EBPα translation by binding to C/EBPα mRNA.[44] The BCR-ABL inhibitor Imatinib downregulates hnRNP-E2 and restores C/EBPα

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protein levels and granulocyte differentiation. C/EBPα serine 21 phosphorylation by ERK1/2 and cyclin-dependent kinase (CDK1) in FLT3 activating mutation is another mechanism for C/EBPα disruption in AML.[45,46] Acetylation of C/EBPα at lysine residues K298 and K302 by general control non-derepressible 5 (GCN5) impairs C/EBPα DNA-binding ability and compromises C/EBPα transcriptional activity.[47] Also, RARα-PLZF found in t(11;17) inhibits C/EBPα by protein interaction and recruitment of histone Deacetylase1 to target loci.[48] In addition to leukemic oncogenes mentioned above, another layer of C/EBPα deregulation in AML is by miR-182, which binds to *CEBPA* 3′-untranslated region, thereby blocking C/EBPα translation.[49] C/EBPα binds to miR-182 promotor and inhibits its expression during granulopoiesis. AML samples with *CEBPA* mutations display elevated levels of miR-182, suggesting C/EBPα-miR-182 feedback loop is critical for proper C/EBPα regulation.

Epigenetic modifications of the *CEBPA* promotor have been found as another major mechanism for C/EBPα inactivation in AML. PMR-RARα, the leukemic fusion protein observed in acute promyelocytic leukemia with t(15;17) deregulates C/EBPα accompanied by DNA methylation.[50,51] *CEBPA* distal promotor methylation associated with decreased C/EBPα expression has been reported in 38% in normal karyotype AML.[52,53] Taken together, these findings show that deregulation of C/EBPα function acts as a major step for myeloid leukemia development. Multiple mechanisms for inhibiting C/EBPα function in leukemia are shown in Figure 1.

CEBPα MUTATIONS IN AML

The role of C/EBPα as a tumor suppressor is underlined by the discovery that *CEBPA* is mutated in AML.[54,55] *CEBPA* is mutated in around 11% of AML patients.[56] The mutations reported in *CEBPA* are point mutations at a bZIP (mentioned as C/EBPα-BRM) and a frame-shift mutation at N-terminus, resulting in a 30 kD isoform of C/EBPα (C/EBPα-p30).[54] *CEBPA* mutations are mostly bi-allelic with one mutation located in N-terminus and other mutation in C-terminus. Absence of homozygous N- or C-terminal mutations suggest that the two mutations possess different functions in leukemogenesis. Studies from multiple laboratories have shown that C/EBPα-p30 is the founder mutation, followed by point mutations in the basic region of C/EBPα. Patients undergoing AML relapse retain the same mutation pattern in both *CEBPA* alleles in comparison to diagnosis, suggesting *CEBPA* mutations are pre-leukemic molecular events in the transformation processes.[57,58] C/EBPα-p42, mutant forms of C/EBPα observed in AML and their functional characteristics are depicted in Figure 2a.

CEBPα MUTATIONS AND LEUKEMIA-INITIATING CELLS

Leukemia-initiating cells (LIC) can originate from hematopoietic stem cells or progenitors with an altered self-renewal function during the transformation process. Proof of *CEBPA* mutations activating self-renewal in committed myeloid progenitor cells in a genetic model was provided by Nerlov and colleagues.[34] By using a non-conditional mouse model for *Cebpa-p30*, they demonstrated that C/ebpa-p30 is able to induce differentiation to committed myeloid progenitors (cMP). This basal differentiation provided by Cebpa-p30, not the loss

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of expression of Cebpa-p42, can act as a platform for induction of myeloid leukemia. Mouse models for *Cebpa* basic region mutants display increased proliferation of long-term HSCs (LT-HSC) and skewed lineage programing of HSCs, resulting in pre-malignant expansion of multipotent progenitors (MPP).[35] In addition, this study demonstrated that combining the N- and C-terminal *Cebpa* mutations in mouse models accelerate leukemia development in comparison to a single mutation, reproducing the bi-allelic mutation pattern seen in patients with *CEBPA* mutations. These two seminal works bring several key points to the field. (1) Loss of C/EBPα-mediated cell cycle control is instrumental in the transformation process of myeloid leukemia. (2) Leukemic stem cells (LSC) in *CEBPA* mutations do not display features of HSC or MPP, rather a c-kit+Mac-1+ committed myeloid progenitor observed for mixed lineage leukemia (MLL).[59] (3) Even though expressed at low levels, C/EBPα plays a major role in HSC lineage priming for downstream myeloid commitment. (4) N-terminal and C-terminal mutations of *CEBPA* have different functions ,which are instrumental in the leukemic transformation. Collectively, these findings suggest mutations of *CEBPA* can convert HSCs to a pre-leukemic state and a downstream progenitor compartment can act as the target for secondary mutations, triggering transformation. Patterns of differentiation block by C/EBPα mutants in comparison to C/EBPα deletion are shown in Figure 2b.

C/EBPα MUTANTS AND MECHANISM OF ACTION

Several models have been suggested for the mechanism of action of C/EBPα-p30. The first model suggested C/EBPα-p30 hetero-dimerize with C/EBPα-p42 and inhibit DNA binding to C/EBPα-p42. This possibility seems less likely, as C/EBPα-p30 with mutations in the heterodimerization domain, which cannot bind C/EBPα-p42, can still block granulocyte differentiation.[60] However, it is clear that C/EBPα-p30 can inhibit C/EBPα-p42 DNA binding, at least on certain targets.[54] Another potential mechanism for the C/EBPα-p30--mediated differentiation block is differential regulation of target genes by C/EBPα-p30. An earlier report suggested C/EBPα-p30 has quite distinct functional properties compared with the C/EBPα-p42 in the lung.[61] We have reported that C/EBPα-p30, but not C/EBPα-p42, upregulates PIN1 as well as UBC9, which in turn blocks C/EBPα-p42 function by multiple mechanisms in AML with *CEBPA* mutations[62,63] Trib2 blocks C/EBPα by proteasome-dependent degradation and is overexpressed in AML with *CEBPA* mutations.[64] Even though both C/EBPα-p42 and C/EBPα-p30 bind to the *Trib2* promotor, they have opposite effect in *Trib2* transcription, suggesting differential effects in transcription.[65] Since both C/EBPα-p42 and C/EBPα-p30 have the same DNA-binding motif, a mechanistic explanation for the selective binding of C/EBPα-p30 to target promotors remains unclear. One possibility is that C/EBPα-p42 and C/EBPα-p30 proteins recruit different protein complexes to target promotors, which can have distinct transcriptional signatures. Careful molecular studies are needed to determine C/EBPα-p30 mediated transcriptional regulation.

CEBPα MUTATIONS AND COOPERATING MUTATIONS

Cancer, including leukemia, results from sequential accumulation of multiple mutations in a specific cell lineage. Gene-expression studies indicated that bi-allelic; but not mono-allelic *CEBPA* mutant samples have distinctive gene-expression profiles[66] that can be predicted by a unique gene signature.[67] The major cooperating molecular abnormalities reported in

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CEBPA mutations are mutations in *GATA2, TET2, ASXL1, WT1, RUNX1* and *FLT3*.[56],[68] Recent reports show mutations in JAK-STAT pathway members such as *CSF3R* and *STAT5B* are frequent in AML with *CEBPA* mutations.[69,70] The most common mutations associated with bi-allelic *CEBPA* mutations and their frequencies are shown in Figure 2c. Even though the N-terminal mutation, resulting in C/EBPα-p30, is known as the founding mutations, our understanding of secondary transformation events in *CEBPA* mutations is limited. A recent study showed that internal tandem duplications in the *Flt3* gene in combination with *CEBPA* mutation result in expansion of the MPP compartment and generation of leukemia-initiating GMPs.[71] This study demonstrated that *Flt3* mutants cooperate with *CEBPA* mutant to induce leukemia in mice. Further studies are needed to elucidate the function of other cooperating events in *CEBPA* mutations, its impact on leukemic stem cell activity and determine if C/EBPα mutants play a role in the acquisition of secondary mutations.

GERMLINE *CEBPα* MUTATION AND CLONAL ORIGIN

Germline *CEBPA* mutations are relatively rare in AML.[72] A recent report found that germline *CEBPA* mutations occur primarily within the N-terminal domain, with acquired (somatic) mutations preferentially targeting the C-terminal.[73] This study also found that *GATA2* mutations are the most common secondary events in germline *CEBPA* mutations. Molecular sequencing of diagnostic and relapsed AML samples with germline *CEBPA* mutations showed leukemic clones that were distinct from the diagnostic AML, suggesting that the recurrences are a second primary leukemia rather than a relapse. Detailed analysis of clonal evolution at relapse showed presence of a novel leukemic clone as well as a sub-clone with mutations in components of DNA hydroxylmethylation such as WT1 and TET2, respectively. This study highlighted that *CEBPA* germline mutations may influence the acquisition and/or selection of cooperating mutations, which may be governed by genetic factors.

REQUIREMENT OF A MYELOID PLATFORM FOR LEUKEMIA INITIATION

Conditional deletion of C/EBPα in mice show a block of myeloid differentiation, but do not develop leukemia.[10] Whether C/EBPα *per se* is required for leukemogenesis was recently debated. Using a transplantation model for *Cebpa* $^{/}$ bone marrow cells transduced with MLL-AF9 retrovirus, Ohlsson and colleagues, reported that C/EBPα is required for the initiation of MLL-driven AML and suggested C/EBPα may collaborate with MLL-ENL.[74] In a related area, Collins and colleagues showed that C/EBPα is essential for the proliferation of Hoxa9/Meis1 transformed cells and that deficiency of C/EBPα displays enhanced survival in primary murine models of Hoxa9/Meis1-induced leukemia.[75] These two papers suggested C/EBPα either collaborate with oncogenes and/or provide enhanced proliferation to leukemic cells, in multiple contexts. These findings have recently been challenged by Ye and colleagues.[76] The study reported that even though C/EBPα deletion results in lack of leukemia development, it can be reverted by inducing myeloid differentiation by cytokines such as GM-CSF and IL-3. This suggests myeloid differentiation rather than the presence of C/EBPα is the requirement for leukemia development.[76] Multiple factors could be contributing to the discrepancy in the

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interpretation of these results. Ye study shows that compared to CMPs, GMPs provide more accessible genomic architecture for oncogenes, suggesting a lack of GMPs could be preventing leukemic transformation in the Ohlsson study. Reduced expression of *Meis1* and *Hoxa9* oncogenes in *Cebpa* deleted MLL-ENL cells, reported by Ohlsson study, could be due to the fact that expression patterns of genes can vary according to the bone marrow compartment and the C/EBPα deletion results in complete block towards GMP. Leukemic cells with RUNX1-ETO and CBFb-SMMHC oncoproteins require wild-type RUNX1 for proliferation and survival, even though both oncoproteins block RUNX1 function.[77,78] So, once transformed MLL leukemic cells could be addicted to C/EBPα, which would be instrumental in the proliferation and survival reported by Collins study. Collectively, these studies show that to initiate myeloid leukemic transformation a differentiation program towards GMP is required, and once the transformation happens, these cells are addicted to key myeloid transcription factors.

THERAPY FOR C/EBPα DEREGULATION IN LEUKEMIA

Being the master regulator in granulopoiesis, restoration of normal C/EBPα levels warrants induction of granulocytic differentiation in leukemic stem and progenitor cells. Several studies demonstrated that restoration of C/EBPα function results in abrogation of leukemia and better survival in leukemic mice.[79,80] 2-Cyano-3, 12-dioxooleana-1, 9-dien-28-oic acid (CDDO), a terpenoid has been shown to increase C/EBPα-p42 protein levels and induces granulocyte differentiation.[81] MEK1/2 inhibitor CI-1040 inhibits hnRNP-E2, which results in enhanced C/EBPα protein levels and granulocytic differentiation.[82] MLN518, a Flt3 tyrosine kinase inhibitor, can induce granulocytic differentiation by inhibiting phosphorylation of C/EBPα on serine 21.[45] Silencing SOX4, a transcription factor overexpressed in bi-allelic *Cebpa* mutation could rescue myeloid differentiation in the humanized mouse model for *CEBPA* mutation, suggesting SOX4 as a potential therapeutic target.[14] Another study of the gene signature in CEBPA dysfunctional AML suggested responsiveness to histone deacetylase inhibitors.[83] High Sensitivity of JAK inhibitors suggest another promising avenue for improved therapeutic strategies for bi-allelic *CEBPA* mutant AML.[69] A recent study showed C/EBPα-p30-dependent inhibition of myeloid differentiation required direct protein–protein interaction with Wdr5, and small molecule-mediated inhibition of this interaction affected the viability of human primary samples with an N-terminal *CEBPA* mutation.[84] Further studies are needed to find therapeutic values of this inhibitor. Discovery of targeted therapeutic approaches such as small molecule-mediated inhibitors that can target C/EBPα-p30 oncogenic function will have great therapeutic value in the treatment of *CEBPA* mutations.

FUTURE DIRECTIONS

Genetic analyses have provided in-depth knowledge to our understanding of leukemia. Significant progress has been made in elucidating transcriptional control in hematopoiesis. However, our understanding of molecular pathogenesis for C/EBPα deregulation in leukemia is limited. Several key questions remain unanswered. (1) What are the molecular mechanisms behind leukemic transformation events during C/EBPα deregulation? (2) How does C/EBPα-p30 selectively bind to target promotors? (3) What are the genes regulated by

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C/EBPα-p30 that play key roles in self-renewal, survival and proliferation? (4) What are the pre-leukemic events that define the pattern for acquisition of secondary mutations? (5) How does clonal evolution of *CEBPA* mutations regulate treatment outcomes in AML? Future studies addressing these questions will provide novel insights, which can be translated to therapeutic approaches.

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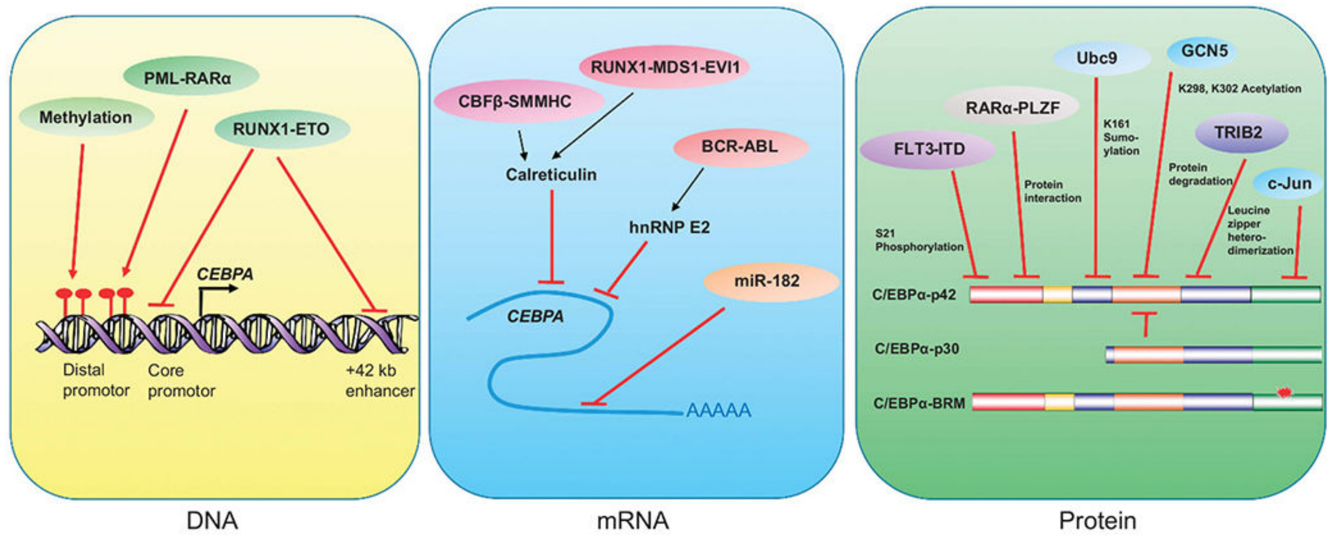

Figure 1.

Multiple mechanisms for C/EBPα inhibition in leukemia. Genetic, epigenetic, transcriptional, translational and post-translational mechanisms of C/EBPα inactivation, which result in block in granulocytic differentiation and leukemia.

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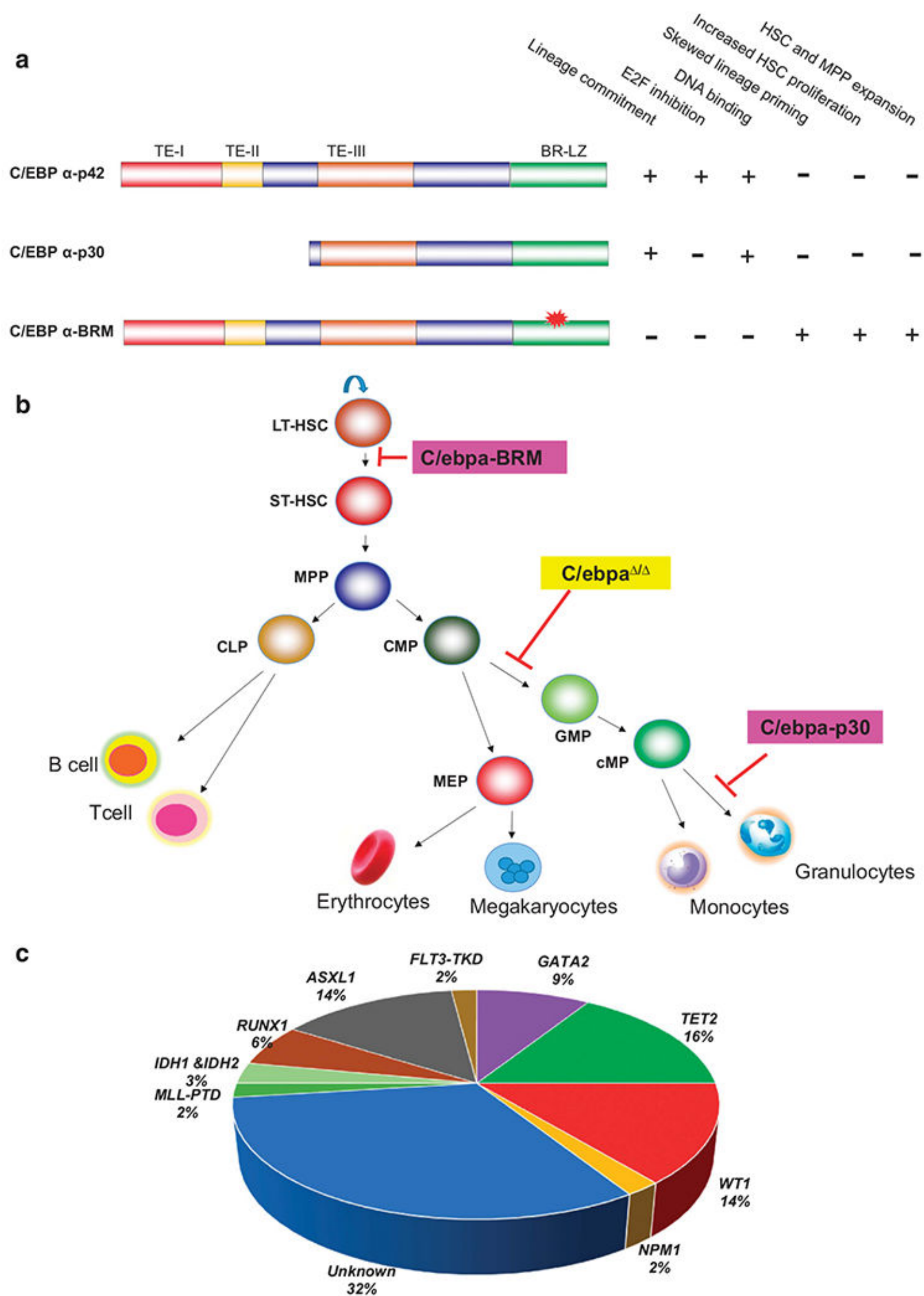

Figure 2.

Characteristics of *CEBPA* mutations in AML. (**a**) Biological roles of C/EBPα-p42 and C/EBPα mutant forms. Three transactivation elements (TE-I, TE-II and TE-III) and a basic-region leucine zipper (BR-LZ) are depicted. C/EBPα-BRM, C/EBPα basic region mutant. (**b**) Distinct differentiation blocks observed during C/EBPα deletion and *CEBPA* mutations. (**c**) Common mutations associated with bi-allelic *CEBPA* mutations (prepared based on Fasan *et al.*,[56]). CLP, common lymphoid progenitors; cMP, committed myeloid progenitors; CMP, common myeloid progenitors; GMP, granulocyte/macrophage progenitors; LT-HSC,

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long-term hematopoietic stem cells; MEP, megakaryocyte/erythroid progenitors; MPP, multi-potential progenitors; ST-HSC, short-term hematopoietic stem cells.