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## C/EBP $\alpha$ in normal and malignant myelopoiesis

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### Abstract

CCAAT/enhancer binding protein  $\alpha$  (C/EBP $\alpha$ ) dimerizes via its leucine zipper (LZ) domain to bind DNA via its basic region and activate transcription via N-terminal trans-activation domains. The activity of C/EBP $\alpha$  is modulated by several serine/threonine kinases and via sumoylation, its gene is activated by RUNX1 and additional transcription factors, its mRNA stability is modified by miRNAs, and its mRNA is subject to translation control that affects AUG selection. In addition to inducing differentiation, C/EBP $\alpha$  inhibits cell cycle progression and apoptosis. Within hematopoiesis, C/EBP $\alpha$  levels increase as long-term stem cells progress to granulocyte–monocyte progenitors (GMP). Absence of C/EBP $\alpha$  prevents GMP formation, and higher levels are required for granulopoiesis compared to monopoiesis. C/EBP $\alpha$  interacts with AP-1 proteins to bind hybrid DNA elements during monopoiesis, and induction of Gfi-1, C/EBP $\epsilon$ , KLF5, and miR-223 by C/EBP $\alpha$  enables granulopoiesis. The *CEBPA* ORF is mutated in approximately 10 % of acute myeloid leukemias (AML), leading to expression of N-terminally truncated C/EBP $\alpha$ p30 and C-terminal, in-frame C/EBP $\alpha$ LZ variants, which inhibit C/EBP $\alpha$  activities but also play additional roles during myeloid transformation. RUNX1 mutation, *CEBPA* promoter methylation, Trib1 or Trib2-mediated C/EBP $\alpha$ p42 degradation, and signaling pathways leading to C/EBP $\alpha$  serine 21 phosphorylation reduce C/EBP $\alpha$  expression or activity in additional AML cases.

### Keywords

C/EBP $\alpha$ ; Myeloid; Differentiation; Acute myeloid leukemia (AML)

### Introduction

CCAAT/enhancer binding protein  $\alpha$  (C/EBP $\alpha$ ), the prototypical basic region-leucine zipper transcription factor, plays a key role during hematopoiesis. Herein, I first review its molecular properties, protein interactions, protein modifications and other levels of post-transcriptional control, and its ability to affect not only differentiation, but also cell proliferation and survival. The role C/EBP $\alpha$  plays during normal myelopoiesis will then be described, including regulation of its expression and activity by other factors, and finally,

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#### Conflict of interest

The author declares that he has no conflict of interest.

the central role that reduced C/EBP $\alpha$  activity or expression plays during myeloid transformation will be delineated.

### Dimerization, DNA-binding, and trans-activation

C/EBP $\alpha$  was initially purified from hepatocytes based on its ability to bind the CCAAT box in the herpes simplex virus thymidine kinase promoter and the enhancer core sequences of simian virus 40, polyomavirus, and murine sarcoma virus [1, 2], followed by isolation of its cDNA from the same source [3]. The C-terminus of C/EBP $\alpha$  was noted to lack helix-breaking glycine and proline residues and to contain leucines spaced every seven residues, predicting formation of an amphipathic  $\alpha$ -helix with a hydrophobic surface capable of mediating dimerization. This domain was designated the leucine zipper (LZ) [4]. Mutagenic analysis revealed that DNA-binding by C/EBP $\alpha$  requires dimerization via its 36-residue LZ followed by site-specific DNA contact by the adjacent 37-residue basic region (BR), the combination also designated the bZIP domain [5, 6]. bZIP domain:DNA co-crystallization and structural analysis confirmed this model of DNA-binding, identifying specific contacts between the C/EBP $\alpha$  BR and the DNA major groove and phospho-ribose backbone [7, 8]. The BR also contains the C/EBP $\alpha$  nuclear localization signal [9, 10].

C/EBP $\alpha$  was further found capable of transcriptional activation of the serum albumin promoter via a specific binding site, dependent upon integrity of an N-terminal and internal trans-activation domains (TADs) [9, 11, 12], as diagrammed (Fig. 1). Interactions of co-activators or co-repressors with C/EBP $\alpha$  have not been extensively characterized, although the SWI/SNF complex was found to contact a central TAD to facilitate gene activation [13], the TIP60 histone acetyltransferase binds C/EBP $\alpha$  to increase trans-activation [14], and DEK, a protein that interacts with histone modifiers, mediates phosphorylation-dependent activity of the N-terminal C/EBP $\alpha$  TAD [15]. C/EBP $\alpha$  can also impact gene expression independent of DNA-binding; for example, interaction of the non-DNA contact surface of its BR with E2F1 reduces c-Myc transcription [16, 17], and C/EBP $\alpha$  displaces HDAC1 or HDAC3 from chromatin-bound NF- $\kappa$ B p50 to activate *Bcl2*, *Flip*, or *Nfkb1* gene transcription [18–20].

The bZIP family of TFs contains three major sub-families, the C/EBP proteins that in addition to C/EBP $\alpha$  include C/EBP $\beta$ , C/EBP $\delta$ , C/EBP $\epsilon$ , C/EBP $\gamma$ , and CHOP, the AP-1 proteins that include c-Fos, c-Jun and related proteins, and the CREB/ATF proteins. The C/EBPs readily homo- or hetero-dimerize via their LZ domains to bind to the DNA element 5'-T(T/G)NNGNAA(T/G) with similar affinity [21, 22], Jun and Fos proteins heterodimerize to bind the AP-1 consensus site 5'-TGA(C/G)TCA, and CREB/ATF proteins homo- or hetero-dimerize to bind the DNA element 5'-TCAGCTGA. AP-1 proteins also heterodimerize with small Maf proteins to bind an extended site, 5'-TGA(C/G)TCAGCA [23]. If one designates the repeating  $\alpha$ -helical residues in the LZ as *abcdefg*, the leucines occupy position *d*, and other hydrophobic residues occupy position *a*, creating a hydrophobic surface that assists dimerization but with low affinity. Salt bridges between positively or negatively charged *e* and *g* residues strengthen the interaction and account for dimerization specificity [24]. C/EBP and AP-1 but not Maf proteins also hetero-dimerize, with reduced affinity compared with C/EBP $\alpha$  homodimers, to bind hybrid DNA elements [25, 26], and C/EBP:ATF hetero-

dimerization also occurs [27], further extending the range of *cis* elements bound by C/EBP proteins.

### Translational, protein modification, and miRNA control

In addition to translation of the dominant 42-kd C/EBP $\alpha$  isoform from a canonical N-terminal AUG, use of an internal AUG leads to expression of a 30-kd isoform lacking the N-terminal TAD [28, 29]. In addition, an extended-C/EBP $\alpha$  46-kd isoform initiating from a non-canonical upstream CUG/GUG contains a nucleolar-localization motif and interacts with nucleophosmin [30]. A conserved upstream open reading frame (uORF) located between this non-canonical translation initiation site and that corresponding to the 42-kd isoform, but with a different reading frame, is thought to control the ratio of p42 vs. p30 translation, dependent upon mTOR activation of eIF-4E and PKR inhibition of eIF-2 $\alpha$ , with increased initiation from the uORF due to reduced PKR or increased mTOR activity leading to increased p30 translation [31, 32]. In addition, calreticulin interacts with GCN nucleotide repeats in *Cebpa* RNA to inhibit its translation [33].

ERK binds an FXFP motif and phosphorylates C/EBP $\alpha$  on S21, near but upstream of the N-terminal TAD, to reduce C/EBP $\alpha$  trans-activation activity, consequent in part to reduced DEK interaction [15, 34]. GSK-3 phosphorylates T222 and T226, dependent on S230 phosphorylation to stimulate C/EBP $\alpha$  activity [35], phosphorylation of S248 via Ras-dependent PKC $\delta$  activation increases C/EBP $\alpha$  trans-activation and is required for induction of 32Dcl3 granulocytic differentiation [36], and PKC $\delta$  modifies additional residues, with S299 modification capable of attenuating C/EBP $\alpha$  DNA-binding [37].

C/EBP $\alpha$  contains a conserved motif IKQEP, with K159 modification by SUMO-1 reducing C/EBP $\alpha$  activity via increased HDAC3 interaction [8–41]. Known sites of C/EBP $\alpha$  protein modification, along with its protein:protein interactions, are diagrammed (Fig. 1).

MicroRNA-690 directly targets *Cebpa* RNA to reduce its expression in a myeloid-derived suppressor cell subset [42], and the Trib1 or Trib2 adaptor proteins facilitate COP1 E3 ubiquitin ligase-mediated C/EBP $\alpha$  degradation, preferentially of the 42 kd isoform [43–45].

### Regulation of cell proliferation, survival, and quiescence

The finding that mature hepatocytes express higher levels of C/EBP $\alpha$  than hepatoma cells provided the first indication that C/EBP $\alpha$  might negatively control cell proliferation [11]. This author's finding that wild-type C/EBP $\alpha$  but not variants incapable of DNA-binding suppress 3T3-L1 preadipocyte colony formation prompted further experiments with estradiol-regulated C/EBP $\alpha$ -ER that revealed direct inhibition of 3T3-L1 cell cycle progression [46]. C/EBP $\alpha$  inhibits proliferation via induction of p21, via interaction of residues 175–187 with CDK2 and CDK4, and via interaction of the outer surface of its BR with E2F proteins, the latter mechanism most active in myeloid cells [16, 47–49]. In hepatocytes, PI3K/AKT mediated S193 dephosphorylation reduces Cdk2/Cdk4 interaction [50]. Of note, mice lacking C/EBP $\alpha$  residues 180–194 have no abnormalities and their fetal liver cells display normal proliferative parameters [51]. C/EBP $\alpha$  also induces miR-34a, which targets E2F3 to limit myeloid cell proliferation [52].

C/EBP $\alpha$  slows apoptosis of the Ba/F3, 32Dcl3, and HF-1 hematopoietic cell lines upon cytokine withdrawal, correlated with increased Bcl-2 and FLIP expression, and C/EBP $\alpha$  or an LZ mutant incapable of DNA-binding directly interacts with NF- $\kappa$ B p50 to bind chromatin and induce Bcl-2 expression and promoter activity, whereas a BR mutant does not bind p50 or induce Bcl-2 [18, 19]. C/EBP $\alpha$  or C/EBP $\beta$  have much higher affinity for NF- $\kappa$ B p50 than they do for NF- $\kappa$ B p65, allowing these C/EBPs to displace HDAC1 or HDAC3 from chromatin-bound p50 to induce NF- $\kappa$ B target genes even in the absence of canonical NF- $\kappa$ B activation [20, 53].

Finally, indicating a role for C/EBP $\alpha$  in maintaining stem cell quiescence, Mx1-Cre-mediated *Cebpa* ORF deletion in adult mice draws long-term hematopoietic stem cells (LT-HSC) into cell cycle, induces apoptosis, and leads to stem cell exhaustion in competitive transplantation assays [54, 55].

### Regulation of normal myelopoiesis

**Expression**—C/EBP $\alpha$  is abundant in several cell lineages, including adipocytes, hepatocytes, and type II pneumocytes [56, 57]. Within hematopoiesis, C/EBP $\alpha$  is preferentially expressed in the granulocyte, monocyte, and eosinophil as compared to the lymphoid or megakaryocyte/erythroid lineages [58, 59]. Amongst marrow stem/progenitor cells, low level *Cebpa* RNA expression is detected in Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> (LSK) cells, increases twofold as these progress to the common myeloid progenitor (CMP) and tenfold further as CMP develop into GMP [60]. *Cebpa* is detectable as well in LT-HSC defined by the LSK/SLAM surface markers [54].

**Consequence of reduced expression**—*Cebpa*<sup>-/-</sup> mice are neonatal lethal due to hepatic dysfunction, but display impaired myelopoiesis [61, 62]. *Cebpa*<sup>(-/-)</sup> fetal liver or marrow from *Cebpa*(f/f);Mx1-Cre adult mice exposed to pIpC to induce Cre expression and biallelic deletion of the *Cebpa* ORF have markedly reduced GMP and myeloid colony-forming units (CFU), with increased CMP, LSK, and megakaryocyte/erythroid progenitors (MEP), and their peripheral blood has markedly reduced neutrophils and monocytes and absence of eosinophils, with twofold elevated platelets and mild lymphocytosis [60, 63]. Beyond the GMP, threefold *Cebpa* knockdown prevents granulopoiesis but not monopoiesis, while sixfold knockdown prevents commitment to either lineage and increases erythropoiesis [64].

**Contribution to monopoiesis**—Reduced levels of C/EBP $\alpha$  may contribute to monopoiesis by hetero-dimerizing with AP-1 proteins such as c-Jun and c-Fos via their respective LZ domains followed by binding to hybrid C/EBP:AP-1 DNA sites, 5'-TGA(C/T)GCAA, commonly found in regulatory elements of genes expressed specifically in monocyte/macrophages and in the *FosB* gene promoter. These hybrid elements often co-localize with PU.1-binding sites; in contrast, PU.1 sites in B cell-specific genes are not found near C/EBP:AP-1 hybrid sites [26, 65]. Use of artificial acidic and basic LZs to direct specific hetero-dimer formation revealed that C/EBP $\alpha$ :c-Fos or C/EBP $\alpha$ :c-Jun but not C/EBP $\alpha$ :C/EBP $\alpha$  or c-Jun:c-Fos complexes direct monocytic commitment of murine myeloid progenitors [25]. Induction of monopoiesis by exogenous C/EBP $\alpha$  may reflect its interaction

with endogenous AP-1 proteins, as a variant harboring the GCN4 LZ was inactive [66–68]. Consistent with fewer but still evident *e/g* LZ salt bridges, C/EBP:AP-1 affinity is approximately twofold weaker than C/EBP:C/EBP affinity, and semi-quantitative Western blot analysis of C/EBP and AP-1 proteins in myeloid cell lines indicates that hetero-dimers could readily form, as was detected by oligonucleotide pull-down, and may be favored by AP-1 protein induction during monopoiesis [26].

**Regulation of granulopoiesis**—Formation of active C/EBP $\alpha$  homo-dimers may be a prerequisite for granulopoiesis. C/EBP $\alpha$  induces transcription of several regulatory proteins required for subsequent lineage maturation, including the transcription factors C/EBP $\epsilon$ , Gfi-1, and KLF5 [64, 69–72]. Absence of C/EBP $\epsilon$  leads to secondary granule deficiency [73], Gfi-1(–/–) mice develop severe neutropenia [74, 75], and KLF5 contributes to 32Dcl3 granulopoiesis [76]. In addition, C/EBP $\alpha$  induces miR-223, leading to degradation of NFI-A mRNA to enhance granulopoiesis [77], and C/EBP $\alpha$  induces miR-30c, which down-regulates Notch1 expression to again favor neutrophilic lineage specification [78]. In addition, C/EBP $\alpha$  cooperates with PU.1, c-Myb, and RUNX1 to activate genes such as myeloperoxidase, neutrophil elastase, lysozyme, lactoferrin, G-CSF receptor (GCSFR), M-CSF receptor (MCSFR), and GM-CSF receptor in immature or mature granulocytic or immature monocytic cells, as previously reviewed [79, 80].

**Regulation of *Cebpa* gene expression during myelopoiesis**—By sorting Lin<sup>–</sup>Sca-1<sup>–</sup>c-Kit<sup>+</sup> marrow cells into GCSFR<sup>+</sup>MCSFR<sup>–</sup> vs. GCSFR<sup>–</sup>MCSFR<sup>+</sup> subsets we enriched for CFU-G vs. CFU-M, demonstrating 2.5-fold increased *Cebpa* RNA in CFU-G [64]. In addition, *Runx1* was enriched 1.5-fold, *Gfi1* fivefold, *Cebpe* 14-fold, and *Klf5* eightfold in CFU-G, whereas *Irf8* was enriched fourfold and *Klf4* twofold in CFU-M, and *PU.1* levels were similar in both. Thus, induction of *Cebpa* transcription to favor C/EBP $\alpha$  homodimer over C/EBP $\alpha$ :AP-1 heterodimer formation may contribute to granulocyte lineage specification.

C/EBP $\alpha$  auto-activates its own promoter, and RUNX1 activates the *Cebpa* promoter modestly, via two conserved non-consensus binding sites, and strongly activates an evolutionarily conserved, 450 bp +37 kb *Cebpa* enhancer, via four consensus RUNX1 *cis* elements [81, 82]. ChIP-Seq data demonstrates that the *Cebpa* enhancer binds RUNX1 as well as SCL, GATA2, LMO2, LYL1, PU.1, Erg, Fli-1, HoxA9, Meis1, Gfi-1b, and C/EBP $\alpha$  [83–85], and notably RUNX1 binding was not evident elsewhere in the *Cebpa* locus [85]. The enhancer also binds p300 and contains the enhancer-specific H3K4me1 histone modification [82]. In transgenic mice in which the +37 kb *Cebpa* enhancer and 845 bp promoter directs expression of a cytoplasmically truncated hCD4 reporter, surface marker analysis and CFU assays demonstrate that the *Cebpa* enhancer/promoter regulatory elements are preferentially active in myeloid compared to lymphoid or erythroid progenitors. In addition, competitive transplantation and FACS analyses demonstrate reporter activity in phenotypic and the large majority of functional LT-HSC [86]. And consistent with these findings, sorting of CMP or LSK/SLAM LT-HSC into hCD4<sup>+</sup> and hCD4<sup>–</sup> subsets revealed that endogenous *Cebpa* mRNA is highly enriched in hCD4<sup>+</sup> CMP [86] or LT-HSC (unpublished). Related results were obtained with another mouse model in which a cDNA

encoding Cre recombinase was inserted into the *Cebpa* locus, followed by breeding to a strain that expresses YFP only in the presence of Cre [87].

LEF-1, reduced in cases of severe congenital neutropenia, activates *Cebpa* gene expression via a binding site located in its promoter region [88], whereas HIF-1 $\alpha$  represses *Cebpa* transcription via an additional promoter site [89], although HIF-1 $\alpha$  may also augment myeloid differentiation via direct interaction with C/EBP $\alpha$  [90]. Finally, a 4.5 kb nuclear, polyA(-), coding-strand RNA encompassing the 2.6 kb *Cebpa* mRNA interacts with DNMT1 via stem-loop RNA structures to limit *Cebpa* promoter methylation and increase gene expression [91].

**Regulation of C/EBP $\alpha$  activity during myelopoiesis**—G-CSF or M-CSF signals direct lineage choice of single, sorted GMP [92]. Stimulation of Lin<sup>-</sup> marrow myeloid progenitors with G-CSF preferentially activates STAT3 and SHP2, whereas M-CSF more potently activates PLC $\gamma$ , PKC, and ERK [93]. As noted, ERK phosphorylates C/EBP $\alpha$  S21 to reduce its activity [34], and M-CSF but not G-CSF increases phospho-S21-C/EBP $\alpha$  in Lin<sup>-</sup> marrow cells, dependent on ERK activation [93]. A homo-dimer of unmodified C/EBP $\alpha$ , expressed at increased levels due to *Cebpa* gene induction by RUNX1 and additional factors, might be required to mediate granulopoiesis, while a phospho-S21-C/EBP $\alpha$ :AP-1 hetero-dimer might still be capable of activating monocyte/macrophage-specific genes in cooperation with PU.1 (Fig. 2). ERK stabilizes c-Fos and induces AP-1 gene induction via Ets:SRF:SRF ternary complex activation to potentially further facilitate monopoiesis while impeding granulocyte lineage development [94]. The p38 MAP kinase can also modify C/EBP $\alpha$  S21 to impede neutrophil development [95], and an MKK6-p38MAPK pathway induces C/EBP $\alpha$  proteasomal degradation to facilitate trans-differentiation of inflammatory neutrophils to monocytes [96].

The SHP2 tyrosine phosphatase inactivates IRF8 [97], a transcription factor required for monopoiesis, and acts on RUNX1 to increase its ability to generate megakaryocytes and CD8 T cells [98]. Induction of SHP2 phosphorylation by G-CSF might occur secondary to activation of Src kinases [99], and we find that tyrosine phosphorylation of Runx1 by Src, or potentially additional kinases, increase its trans-activation potency to facilitate granulopoiesis (unpublished). As noted, sumoylation of C/EBP $\alpha$  reduces its activity, and hypo-sumoylation increases myelopoiesis in zebrafish embryos [100].

**Cross-talk with additional transcription factors**—PU.1, an Ets transcription factor, is also a key regulator of myeloid development. *PU.1*<sup>-/-</sup> mice lack B cells and monocytes and have markedly reduced neutrophils [101]. In contrast to C/EBP $\alpha$ , higher levels of PU.1 are required for monocyte as compared to granulocyte lineage development; Mx1-Cre mediated deletion of the 236 bp -14 kb *PU.1* enhancer leads to 80 % reduction of PU.1 with loss of monopoiesis but preservation of granulopoiesis [102]. RUNX1 also contributes to myelopoiesis. The -14 kb *PU.1* and +37 kb *Cebpa* enhancers are each activated by RUNX1, with exposure of *Runx1*(f/f);Mx1-Cre mice to pIpC reducing *Cebpa* mRNA twofold and PU.1 mRNA 1.5-fold in CMP and GMP [82, 103]. Notably, from a functional perspective *Runx1* deletion impairs granulopoiesis while increasing monopoiesis, leading us to propose that *Cebpa* is more critical than *PU.1* as a RUNX1 target during both normal and malignant

myelopoiesis [82]. The PU.1 promoter and –14 kb enhancer are each activated by C/EBP $\alpha$  [104, 105]. The quantitative importance of this regulation during myelopoiesis is uncertain given that higher levels of C/EBP $\alpha$  favor granulopoiesis, whereas higher levels of PU.1 favor monopoiesis. PU.1 induction by C/EBP $\alpha$  could potentially vary during hematopoiesis, e.g. increased at the GMP stage to facilitate myeloid vs. mega-karyocyte/erythroid commitment and then reduced to facilitate granulocyte vs. monocyte lineage specification.

By forming a C/EBP $\alpha$ :c-Jun heterodimer via LZ interaction, C/EBP $\alpha$  can divert c-Jun:c-Fos complexes from auto-activating the c-Jun promoter [106], though we have suggested that during myelopoiesis AP-1 complexes are not depleted by C/EBP proteins, but rather that C/EBP:AP-1 heterodimers binds hybrid DNA elements to activate monocytic genes in cooperation with PU.1 and AP-1 [25]. C/EBP $\alpha$  can directly interact with PU.1 to inhibit activation of a model PU.1 reporter [107], although cooperative activation of myeloid genes such as neutrophil elastase by C/EBP $\alpha$  and PU.1 indicates that this mechanism is not always operative [108]. IRF8 directly interacts with C/EBP $\alpha$  to inhibit its interaction with endogenous C/EBP $\alpha$  target genes, thereby preventing granulopoiesis to favor monopoiesis [109]. Perhaps C/EBP $\alpha$ :AP-1 hetero-dimers are less sensitive to IRF8 inhibition, allowing them to contribute to monopoiesis in the presence of IRF8. C/EBP $\alpha$  down-regulates *Pax5* RNA through an uncertain mechanism to inhibit lymphopoiesis [110]. Exogenous C/EBP $\alpha$  impairs and reduced C/EBP $\alpha$  enhances erythropoiesis [60, 63, 64, 70]. The effect of reduced C/EBP $\alpha$  on erythropoiesis might reflect decreased PU.1 levels, leading to GATA-1 derepression [111, 112]. However, *Cebpa* knockdown only reduced PU.1 1.6-fold while markedly enhancing erythropoiesis, leading to the speculation that direct regulation of GATA-1 or a GATA-1 co-factor by C/EBP $\alpha$  might also restrict the CMP to MEP transition [64]. Indeed avian C/EBP $\beta$  down-regulates *FOG* mRNA to enable eosinophil lineage development [113]. Of note, induction of GATA-2 in C/EBP $\alpha$ -expressing GMP leads to eosinophil lineage commitment [59]. Finally, mice lacking the bHLH transcription factor Twist-2 have increased neutrophils, monocytes, and basophils, in part reflecting the ability of Twist-2 to inhibit C/EBP $\alpha$  trans-activation [114].

### Role of reduced C/EBP $\alpha$ expression or activity in myeloid transformation

**CEBPA ORF mutations**—The protein-coding *CEBPA* ORF is mutated in approximately 10 % of AML cases, most often those with FAB M1 or M2 morphology lacking t(8;21) [115, 116]. Two categories of mutations occur. N-terminal mutations lead to premature translational termination of C/EBP $\alpha$ <sub>p42</sub> and increased levels of C/EBP $\alpha$ <sub>p30</sub>, lacking a TAD. C-terminal mutations occur in the LZ, often in its first  $\alpha$ -helix, preventing DNA-binding. About 50 % of AMLs with *CEBPA* ORF mutations have an N-terminal mutation on one allele and a C-terminal mutation on the other, and patients with double-mutant *CEBPA* have improved prognosis compared to those with only one mutant allele [117]. Approximately 10 % of AML cases with *CEBPA* N-terminal mutations acquire the alteration via the germline [117]. Less than 5 % of patients with myelodysplastic syndrome (MDS) harbor *CEBPA* mutations, and these are most often mono-allelic [116, 118, 119].

The large majority of C-terminal mutations are in-frame, indicating that these C/EBP $\alpha$ <sub>LZ</sub> variants contribute to transformation not only due to their lack of DNA-binding activity, but

also as active oncoproteins. Both C/EBP $\alpha$ p30 and several C/EBP $\alpha$ LZ mutants retain the ability to interact with NF- $\kappa$ B p50 to induce Bcl-2 and inhibit apoptosis [18, 19, 53], a C/EBP $\alpha$ LZ variant interfered with the ability of C/EBP $\alpha$ p42 to activate transcription in cooperation with PU.1 [119], potentially reflecting sequestration of PU.1 or co-activators, and additional protein interactions might further allow C/EBP $\alpha$ LZ variants to contribute to transformation.

The ability of C/EBP $\alpha$ p42 to inhibit cell cycle progression consequent to interaction with E2F proteins is dependent upon integrity of its N-terminus [16]; therefore, C/EBP $\alpha$ p30 likely has reduced ability to inhibit proliferation of leukemic blasts via effects on cell cycle regulator proteins, as is observed [120, 121]. C/EBP $\alpha$ p30 can zipper with C/EBP $\alpha$ p42 and weaken its trans-activation strength, and p30:p30 homodimers compete with p42:42 homodimers for binding to DNA [115]. p30 binds a subset of C/EBP sites with reduced affinity compared with p42 but binds others with equal affinity, retains a TAD and induces multiple genes not affected by p42, including the Ubc9 SUMO ligase, and may further alter progenitor biology via protein interactions [115, 121–124].

Heterozygous WT/p30 knockin mice have no hematologic abnormalities; in contrast p30/p30 mice uniformly develop AML by one year [120]. Preleukemic p30/p30 mice are neutropenic but retain GMP and myeloid CD11b<sup>low</sup>c-kit<sup>+</sup> CFUs that replat indefinitely in IL-3/SCF. Biallelic C/EBP $\alpha$ LZ mice lack GMP and only develop delayed erythroleukemia, whereas LZ/p30 mice develop myeloid transformation with more rapid kinetics than p30/p30 mice [125]. C/EBP $\alpha$ p30 apparently provides C/EBP $\alpha$  activity sufficient to generate GMP but insufficient for further myeloid maturation, and these GMP are then subject to further mutations leading to full myeloid transformation. In contrast, *Cebpa*( $-/-$ ) mice do not develop GMP or AML, but have increased CMP and LSK [60], consistent with the finding that p30/p30 or LZ/p30 leukemia-initiating cells (LIC) reside predominantly in the GMP or Lin<sup>lo</sup>Sca-1<sup>-</sup>c-kit<sup>+</sup>CD11b<sup>lo</sup> populations [120, 121]. Of note, in the absence of C/EBP $\alpha$ , neither Bcr-Abl, MLL-ENL, nor HoxA9/Meis1 induce myeloid transformation, consistent with the idea that a minimal level of C/EBP $\alpha$  activity is required to generate GMP as a substrate for transformation [85, 126, 127]. Consistent with this idea, co-expression of the FLT3ITD-activated tyrosine kinase receptor with C/EBPp30 and a C/EBP $\alpha$ LZ mutant accelerates transformation, with preleukemic GMP expansion [128].

In contrast to C/EBPp30, any one of several C/EBP $\alpha$ LZ variants block 32Dcl3 myeloid differentiation, induce marrow CFU replating upon retroviral transduction, and induce AML upon transplantation of transduced marrow cells, alone and accelerated by FLT3ITD [119, 129]. In addition, C/EBP $\alpha$ LZ variants reduce *Mcsfr/Csflr* expression, and ectopic *Csflr* cooperates with a C/EBP $\alpha$ LZ variant harboring a C-terminal LZ mutation to induce myeloid transformation [129]. In contrast to these findings with C/EBP $\alpha$ p30, LIP, an N-terminally truncated C/EBP $\beta$  isoform, but not full-length C/EBP $\beta$ , induces indefinite myeloid CFU replating and AML in vivo, potentially via C/EBP $\alpha$  inhibition [130].

**RUNX1 down-modulation**—Alterations affecting RUNX1 or its partner CBF $\beta$  are common in AML [131]. 12 % of AMLs harbor t(8;21), leading to expression of RUNX1-ETO, which binds RUNX1 *cis* elements to represses *Cebpa* transcription [132]. 8 % of



AMLs, mainly the M4eo subset, have inv(16), expressing CBF $\beta$ -SMMHC, which binds RUNX1 to inhibit its activity [133], and 13 % of cases, most often M0, have inactivating point mutations in the RUNX1 DNA-binding domain or TAD [134]. RUNX1 point mutations are also present in about one-third of cases in which patients with the myeloproliferative diseases chronic myeloid leukemia (CML), polycythemia vera, or essential thrombocytopenia have progressed to AML [135, 136], and RUNX1 point mutations are also found at high frequency in therapy-related MDS and in ~3 % of sporadic MDS cases [137]. RUNX1 point mutation most often occurs in its DNA-binding domain on one allele; these variants do not bind DNA, but dominantly inhibit RUNX1 trans-activation, potentially via interference with RUNX1 dimerization and by competition for CBF $\beta$ , a protein required for RUNX1 DNA-binding [137, 138]. CML blast crisis also occasionally manifests t(3;21), expressing RUNX1/MDS1/EVI1 (RME), fusing the RUNX1 DNA-binding domain with MDS1/EVI1 to generate a potent repressor of RUNX1 targets; RME also cooperates with Bcr-Abl to induce AML [139].

Reduced *CEBPA* transcription consequent to each of these RUNX1 alterations, resulting from reduced activity of the *CEBPA* promoter and its +41 kb enhancer (homologous to the murine +37 kb enhancer), may be central to their ability to contribute to myeloid transformation. ChIP-Seq data for RUNX1-ETO from two human AML patient samples and from the Kasumi-1 cell line demonstrates exclusive binding at the +41 kb *CEBPA* enhancer [140]. In contrast, *CEBPA* enhancer point mutations or small deletions were not seen in AML cases [141]. Of note, Mx1-Cre-mediated *Runx1* gene deletion in adult mice does not lead to AML, likely due to modest twofold *Cebpa* mRNA reduction in GMP, whereas active repression of the *Cebpa* +37 kb enhancer by RUNX1-ETO or dominant-inhibition of RUNX1, RUNX2, and RUNX3 by CBF $\beta$ -SMMHC or RUNX1 mutants likely results in further *Cebpa* suppression.

Mice lacking the RUNX1-regulated *PU.1* -14 kb enhancer uniformly develop AML by 6 months [102]. However, *PU.1* gene mutations occur in <1 % of human AML cases [142]. As noted, our study of *Runx1*-deleted mice led us to suggest that reduction in C/EBP $\alpha$  rather than PU.1 activity is the more critical consequence of RUNX1 oncoprotein expression in human AMLs [82].

**Other alterations in AML affecting C/EBP $\alpha$** —Approximately 50 % of AML cases have reduced *CEBPA* mRNA, with about threefold median reduction amongst these cases, maximum tenfold, vs. CD34<sup>+</sup>CD38<sup>+</sup> (mainly myeloid) progenitors [143]. *CEBPA* promoter CpG hyper-methylation, evident in 37 % of AML cases [143], may in part reflect reduced activity of RUNX1 or other transcription factors that normally activate the promoter as well as selection of preleukemic blasts with repressive promoter methylation. Approximately 3 % of AML cases harbor dense *CEBPA* promoter hyper-methylation leading to silencing of *CEBPA* expression and up-regulation of T cell genes [144]; in these cases the gene encoding C/EBP $\gamma$ , located just upstream of the *CEBPA* gene, is markedly induced due to derepression of E2F proteins [145]. The ability of C/EBP $\gamma$  to zipper with other C/EBPs and reduce their trans-activation potency might contribute to myeloid transformation in these cases. Leukemic blasts from patients with silenced *CEBPA* or those with biallelic *CEBPA* ORF mutations have a gene expression profile similar that obtained from the preleukemic LIC

population isolated from mice harboring biallelic C- and N-terminal C/EBP $\alpha$  mutant variants or those with reduced Sox4, a gene directly repressed by C/EBP $\alpha$  [146].

Over-expression of Trib1 or Trib2, which direct C/EBP $\alpha$ 42 but not p30 degradation via the E3 ubiquitin ligase COP1, occurs in 15–20 % of AMLs, and Trib1 or Trib2 are transforming in murine models [43, 147, 148]. FLT3ITD activates ERK and CDK1 to stimulate phosphorylation of C/EBP $\alpha$ S21, reducing C/EBP $\alpha$  trans-activation strength [149, 150], and Bcr-Abl inhibits translation of *Cebpa* mRNA by inducing hnRNP E2 [151]. Pathways known to down-modulate C/EBP $\alpha$  in AML are summarized, along with additional pathway that might be relevant (Fig. 3).

## Conclusions

The ability of C/EBP $\alpha$  to homo-dimerize and also to hetero-dimerize with other bZIP proteins extends the range of DNA elements it interacts with and provides opportunity for regulation of both C/EBP $\alpha$  and its partners. Future investigations should provide further insight into the role these interactions and C/EBP $\alpha$  protein modifications play during myelopoiesis, including determining whether C/EBP $\alpha$  S21 phosphorylation precluded activation of granulocytic genes but still allow induction of monocytic genes as a heterodimer with AP-1 proteins and assessing the role of additional C/EBP $\alpha$  modifications, e.g., lysine acetylation or arginine methylation. The capacity of C/EBP $\alpha$  to inhibit cell proliferation requires unique control during different stages of hematopoiesis to allow the requisite balance between the proliferative drive and induction of lineage-specific genes. For example, GMP harbor high-levels of C/EBP $\alpha$  and yet have high proliferative potential—do they possess a mechanism to suppress C/EBP $\alpha$ -mediated inhibition of G1 to S cell cycle progression? Further elucidating how the N-terminus of C/EBP $\alpha$  contributes to cell cycle inhibition upon BR interaction with E2F proteins might provide relevant insight. Several C/EBP $\alpha$  protein modifications have been identified, but their role during normal myelopoiesis is poorly understood.

Existence of multiple pathways to C/EBP $\alpha$  inhibition supports the idea that reduction of C/EBP $\alpha$  expression or activity is central to the pathogenesis of AML. Perhaps means can be found to reactivate C/EBP $\alpha$  as “differentiation therapy” for AML or MDS, either by inducing expression of normal *CEBPA* alleles, targeting signaling pathways, miRNAs, or non-coding RNAs to favor increased C/EBP $\alpha$  expression or activity, or inducing other C/EBP family members that might substitute for C/EBP $\alpha$ . In particular, C/EBP $\beta$  induces granulopoiesis in response to cytokine signals in the absence of C/EBP $\alpha$ , and replacement of the *Cebpa* ORF by *Cebpb* is well tolerated [152, 153].

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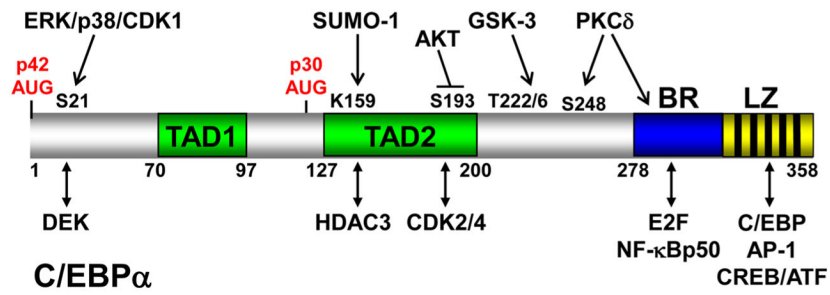
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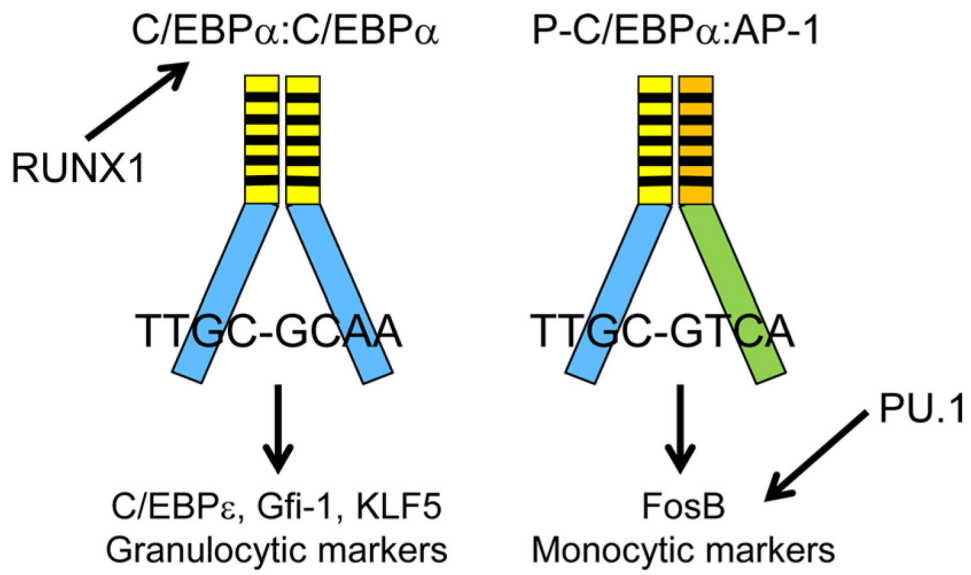
**Fig. 1.** Diagram of C/EBP $\alpha$ , showing the location of its trans-activation domains, basic region, leucine zipper, initiating AUG residues, protein modifications, and protein interactions

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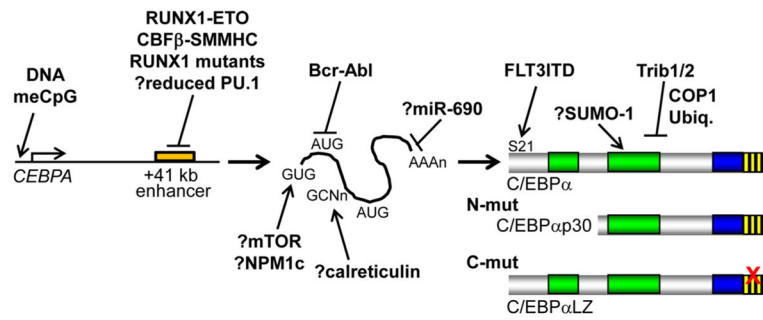
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**Fig. 2.** Diagram of a C/EBP $\alpha$ :C/EBP $\alpha$  homodimer and a C/EBP $\alpha$ :AP-1 heterodimer bound to consensus *cis* elements, with cooperating transcription factors and gene induction events that enable their contribution to granulopoiesis versus monopoiesis



**Fig. 3.** Pathways, both described and potential, that mediate reduced C/EBPα expression or activity in AML cases via effects on *CEBPA* transcription, mRNA translation or stability, or protein expression, stability, or activity