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C/EBPα and MYB regulate FLT3 expression in AML

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Abstract

The interaction between the receptor FLT3 (FMS-like tyrosine kinase-3) and its ligand FL leads to crucial signalling during the early stages of the commitment of haematopoietic stem cells. Mutation or over-expression of the *FLT3* gene, leading to constitutive signalling, enhances the survival and expansion of a variety of leukaemias and is associated with an unfavourable clinical outcome for acute myeloid leukaemia (AML) patients. In this study, we used a murine cellular model for AML and primary leukaemic cells from AML patients to investigate the molecular mechanisms underlying the regulation of *FLT3* gene expression and identify its key cis- and trans-regulators. By assessing DNA accessibility and epigenetic markings, we defined regulatory domains in the *FLT3* promoter and first intron. These elements permit *in vivo* binding of several AML-related transcription factors, including the proto-oncogene MYB and the CCAAT/enhancer binding protein C/EBPα, which are recruited to the *FLT3* promoter and intronic module, respectively. Substantiating their relevance to the human disease, our analysis of gene expression profiling arrays from AML patients uncovered significant correlations between *FLT3* expression level and that of *MYB* and *CEBPA*. The latter relationship permits discrimination between patients with CEBPA mono- and bi-allelic mutations, and thus connects two major prognostic factors for AML.

Keywords

AML; C/EBP alpha; FLT3; MYB; regulation

INTRODUCTION

Acute myeloid leukaemia (AML) represents a biologically and clinically heterogeneous disorder characterised by a clonal expansion of haematopoietic progenitor cells blocked in

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their maturation. About 50% of AMLs present cytogenetic abnormalities of various types that can be used to categorise the patients into different risk groups. The remaining cytogenetically normal AML (CN-AML) can be subdivided based on molecular markers, such as mutations in the genes encoding the FLT3 receptor, C/EBPα, NPM1, IDH1 and IDH2, C-KIT, RAS or WT1.[1]

Constituting some of the most frequent mutations found in CN-AML, mutations in the *FLT3* gene generally associate with an inferior outcome. The FMS-like tyrosine kinase-3 (FLT3), a member of the Type III RTK subfamily, is a membrane-bound tyrosine kinase receptor, which together with its ligand FL, has an important role in the normal development of stem cells and the immune system.[2,3] The FLT3 protein is commonly mutated in AML, with 20–30% of cases carrying an internal tandem duplication within the juxtamembrane domain (*FLT3-ITD*) and 8–12% a single point mutation at position 835 in the kinase domain (*FLT3-D835*), leading to constitutive signalling and enhanced cell survival and proliferation. While the presence of the *FLT3-ITD* mutation significantly correlates with adverse clinical outcome, the prognostic significance of FLT3 single point mutations remains unclear (see[4] for review). By themselves, FLT3-ITD mutant proteins are sufficient to induce a myeloproliferative disorder, but the development of *FLT3-ITD* AML may require additional mutations that impair haematopoietic differentiation.[5]

In this respect, the functional cooperation between *FLT3-ITD* and mutation of the CCATT/enhancer binding protein alpha (C/EBPα) may be of particular significance in CN-AML.[6,7] C/EBPα is a leucine-zipper transcription factor that has a pivotal role in granulocyte and neutrophil development[8] but also act as a tumour suppressor in the haematopoietic system.[9] As the result of an alternative translation initiation, C/EBPα is produced in two major isoforms, p42 (42kDa) and p30 (30kDa). Mutations of the *CEBPA* gene are reported in 5–14% of patients with AML and can be largely categorised into two types: the *N*-terminal stop mutations that result in the expression of the p30 isoform from the mutated allele and the *C*-terminal inframe insertions/deletions within the basic region leucine zipper DNA binding domain.[10,11] In most cases, *CEBPA* mutations are bi-allelic with one allele harbouring the *N*-terminal mutation and the other the *C*-terminal, while the homozygous *N*- or *C*-terminal mutations are relatively rare.[12] Several studies have demonstrated that bi-allelic mutations of the *CEBPA* gene give a favourable prognosis for AML patients,[13] although this benefit appears to be nullified in the presence of FLT3 activating mutations.[14] When coexisting, *FLT3-ITD* mutations may cooperate in leukaemogenesis by over-riding the differentiation impairment brought about by C/EBPα bi-allelic mutations and promote the formation of committed myeloid progenitors, the templates for leukaemia-initiating cells.[6]

In the absence of FLT3 mutation, the auto-activation of the receptor was also reported in patients with high expression level of FLT3, making this feature another poor prognosis marker.[15] As might be expected, the combination of both *FLT3-ITD* mutation and high-level expression associates with a dismal outcome for patients. Paralleling FLT3 activating mutations, constitutive signalling through FLT3 overexpression may cooperate with other mutations in leukaemogenesis. For instance, such collaboration was suggested with RUNX1 mutation in AML with chromosome 13 trisomy.[16] In light of its clinical relevance, it is

surprising that, aside from recent evidence indicating a role for HOXA9 and MEIS1,[17,18] the mechanisms regulating FLT3 expression in AML remains largely undefined.

In order to investigate the regulators of *FLT3* transcription in AML, we made use of a murine-cultured model for CN-AML and bone marrow samples from CN-AML patients. We identified two enhancers regulating *FLT3* expression by allowing recruitment of several AML-related transcription factors. Among these, we identified the transcription factor MYB, which is increasingly implicated as a key component of leukaemia maintenance and oncogene addiction, and C/EBPα as keys regulators of *FLT3* expression in AML. Furthermore, we showed a direct relationship between *CEBPA* mutation status and *FLT3* expression level and highlighted an important molecular connection between these factors in CN-AML cells.

MATERIALS AND METHODS

Cell line

FMH9 cells were cultured in RPMI medium supplemented with 10% foetal bovine serum, 50 ng/ml stem cell factor, 5 ng/ml granulocyte-macrophage colony-stimulating factor, 5 ng/ml interleukin-3 and 5 ng/ml interleukin-6. All the cytokines were purchased from Peprotech EC (London, UK).

AML patient samples

Collected at diagnosis, bone marrow samples of *de novo* AML patients were obtained through the Central England Haemato-oncology Research Biobank and used for Ficoll-Plaque separation of white mononucleated cells.

X-ChIP

X-ChIP assays were performed as previously described,[19] using antibodies from Santa Cruz Biotechnology (Dallas, TX, USA) Upstate Ltd (Merck Millipore, Billerica, MA, USA) (MYB) or locally produced antibodies (H3K9ac, H4K8ac).

Nuclease hypersensitive site mapping

Cell nuclei, prepared in 1 ml digestion buffer (15 mM Tris-HCl pH7.5, 15 mM NaCl, 60 mM KCl, 5 mM $MgCl_2$, 300 mM glucose, 0.5 mM EGTA, 0.1%NP40), were digested 10 min at 37 °C with DNase I (0–60units). The reaction was terminated by adding 330 μl of 100 mM EDTA/4% SDS. RNA and proteins were sequentially digested at 37 °C with 100 μg RNaseA (1 h) and 100 μg proteinase K (overnight). Extracted DNA was used as template for quantitative-PCR reactions (primers listed in Supplementary Table S1).

Patient profiling arrays

AML patient CEL files were analysed using MAS (background), INVARIANT SET (normalisation), MAS (pm correct) and LIWONG (summary) methods as recommend for gene coexpression analysis.[20] Intensities were mean centred to account for undesirable variations (Supplementary Figure S2). The respective scaling factors are listed in Supplementary Table S2. For transcripts represented by multiple probes on the array, the

average value of the different probes was determined. The log10 values of the gene expression from all 46 CN-AML *CEBPA* wild-type profiling arrays in the data set GSE15210 were used for the calculation of the Pearson correlation coefficient. Within the larger GEO data set GSE14468 containing a mix of CN and abnormal AML cases, 58 profiling arrays from CN-AML of M0 to M3 subtypes (according the French-American-British classification system) with no *CEBPA* mutations were identified and used for this study as well as seven CN-AML with a *CEBPA* monoallelic *N*-terminal stop mutation and 25 CN-AML arrays of patients with *CEBPA* bi-allelic mutations.

Plasmids and transfection method

In total, 5×10^6 FMH9 cells were electroporated using the Amaxa transfection kit (Biosystems, Warrington, UK) according to the manufacturer's instructions. The expression vectors for the wild-type form of C/EBPα and MYB as well as the correspondent small hairpin RNA (shRNA) were purchased from Origene (Cambridge, UK). The C/EBPα mutant constructs (Supplementary Figure S3), kindly provided by Toshio Kitamura, were as described.[7]

RESULTS

The FMH9 cell line recapitulates AML cell features

To define the mechanism underlying *FLT3* gene regulation in leukaemia, we used a murine cell line modelling CN-AML. The FMH9 line was obtained from primary bone marrow progenitors cotransduced with HOXA9 and MEIS1, a combination causal for AML.[18,21,22] Parallel morphological and flow cytometric analyses with patient cells confirmed the FMH9 AML-like phenotype (Figure 1a). Expressing the surface markers GR-1, CD11b, KIT and FLT3, the transformed murine myeloblastic FMH9 cells mimic a CD14 + /CD11b + /CD38 + /c-Kit + /Flt3 + leukaemic fraction of CN-AML patient bone marrow (Figure 1b). Crucially, mRNA quantification indicated similar levels of *FLT3* expression in FMH9 and AML patient cells (Figure 1c).

Flt3 cis-regulatory modules lie in the promoter and first intron

The *Flt3* gene is characterised by an abundance of repeated sequences and an overall low degree of cross-species sequence conservation. On the basis that crucial cis-regulatory domains are preserved across species, we focussed on scrutinising the regions of sequence conservation. Three of these regions (A, B and C) locate to the regions surrounding the promoter, the *Flt3* transcription start site and the first intron (Figure 2). Using our murine model for CN-AML, we assayed DNaseI sensitivity across these domains to locate sites of nuclease hypersensitivity, symptomatic of protein-DNA interaction. This analysis highlighted three hypersensitive sites at – 1.4 kb (HSA), –0.15 kb (HSB) and + 7.5 kb (HSC) relative to the *Flt3* initiating codon (Figure 2b). To further assess their potential relevance with regards to *Flt3* transcriptional regulation, we tested for the presence of histone marks that associate with regulatory domains, and found H4K8ac, H3K9ac and H3K4me3 in the vicinity of each domain (Figure 2c).

HOXA9, MEIS1, MYB and C/EBPα bind to the *Flt3* regulatory modules in the FMH9 AML-line

As a first step towards the identification of potential trans-regulators, the sequences encompassing the different hypersensitive sites were screened for the presence of transcription factor binding sites that are conserved across species. This analysis identified several consensus sites for the binding of leukaemia-associated factors such as MYB, C/EBPα and HOX-TALE partner proteins (Supplementary Figure S1). To assess the possible recruitment of factors to the *Flt3* locus, we performed X-ChIP assays, using antibodies directed against the different candidate trans-regulators and analysed the immunoprecipitated material by quantitative-PCR at the sites of hypersensitivity and two control regions (Figure 3). In agreement with the previous works,[17,18] we identified direct binding of HOXA9 and MEIS1 to the *Flt3* promoter in the AML-like FMH9 line, which ectopically expresses both factors. Interestingly, these data also show direct recruitment of MYB and C/EBPα to the *Flt3* promoter and enhancer module, respectively.

FLT3 expression correlates with that of *HOXA9, MEIS1, MYB* and *CEBPA* in CN-AML

The abundance of the aforementioned factors and their potential influence on *FLT3* expression were next evaluated in CN-AML. For this purpose, we made use of publicly available data sets of AML patient profiling arrays and assessed the relationship between the expression of *FLT3* and its predicted regulators. Evaluating gene expression codependency, the Pearson correlation coefficients were calculated between the logarithmic expression of *FLT3* and the different candidate factors. As C/EBPα is included in this list, the analysis was restricted to patients with no *CEBPA* mutations, summing 46 and 58 CN-AML arrays within two independent sets (Geo data sets GSE15210 and GSE14468, respectively). Remarkably, despite the biological heterogeneity and variety of genomic mutations included within and between the sets, the analysis showed that *FLT3* RNA expression is linked to that of *CEBPA, MYB, MEIS1* and *HOXA9* in CN-AML (Figure 4). Together with our finding that all four proteins are recruited to the *Flt3* promoter and intronic regions in the FMH9 line, these data strongly suggest that MYB and C/EBPα have a determining role alongside HOXA9 and MEIS1 in the control of *FLT3* expression in CN-AML.

MYB and C/EBPα bind to the human *FLT3* promoter and intronic elements in CN-AML cells

To confirm that our findings using the FMH9 line were representative of the human disease condition, we reproduced the nuclease assays and X-ChIP experiments using cells from different CN-AML patients. As shown in Figure 5a, the human *FLT3* locus has a large number of insertions compared with the murine locus. However, the three identified regions are found conserved at –15.3 kb, –0.37 kb and + 6.2 kb from the human *FLT3* initiating codon. The intronic region C was split into two regions (hC1 and hC2) by the presence of a repeated sequence. At these locations, DNaseI hypersensitive sites were only consistently found in regions A and C in CN-AML patient cells (Figure 5b). In agreement with our findings using the murine line, X-ChIP experiments demonstrated the binding of MYB and C/EBPα in human AML to regions A and C on the *FLT3* locus, respectively (Figure 5c).

MYB and C/EBPα expression levels influence *Flt3* expression

To investigate the extent to which MYB and C/EBPα contribute to the regulation of *Flt3* transcription, we manipulated their levels through shRNA knockdown and over-expression in the murine FMH9 AML model. Both shRNA-mediated silencing and over-expression of MYB and C/EBPα were achieved by transient transfection, 24 h after which *Flt3* RNA levels were measured by quantitative-PCR. Reduction or over-expression of MYB led to a parallel down- and upregulation of *Flt3* expression in the AML-like cells (Figure 6 upper panels). In contrast, while C/EBPα silencing also led in a decrease in *Flt3* RNA levels, enforced C/EBPα expression only resulted in a moderate elevation of its transcript accompanied by a marked downregulation of *Flt3* expression (Figure 6 lower panel). This latter result suggests that regulation of *Flt3* expression could depend on strict C/EBPα activity thresholds in AML.

FLT3 expression is affected in CN-AML with *CEBPA* bi-allelic mutation

The latter finding raised the question of the impact that *CEBPA* mutations may have on *FLT3* expression regulation in CN-AML. To address this question, we assessed and compared the relationship between *FLT3* and *CEBPA* expression in patients with different *CEBPA* status. Pearson correlations were determined using expression profiling arrays of CN-AML with wild-type *CEBPA, CEBPA* monoallelic *N*-terminal stop mutation (expressing the C/EBPα p30 isoform) and *CEBPA* bi-allelic mutations. The results revealed that the log linear relation between *FLT3* and *CEBPA* transcript expression were conserved in patients with a *CEBPA* mono-allelic *N*-terminal stop mutation but lost in the presence of *CEBPA* bi-allelic mutations (Figures 7a and b). As the loss of this relationship would be consistent with an impairment of C/EBPα-mediated *FLT3* transcriptional regulation, we proceeded to evaluate how *FLT3* expression may be affected in this subgroup of patients by plotting and comparing *FLT3* transcript levels in patients with different *CEBPA* status (Figure 7c). This analysis showed that *CEBPA* bi-allelic mutations associate with a decrease in the median expression level of *FLT3*.

The signature of FLT3 constitutive activation is largely inverted in CN-AML with *CEBPA* bi-allelic mutation

To test whether this reduced *FLT3* expression could affect the receptor function and assess how this may compare with the effects of the *FLT3-ITD* activating mutation, we analysed the expression of genes that were shown to be downstream targets of FLT3-ITD signalling (Figure 8a) and components of the *FLT3-ITD* molecular signature in CN-AML (Figure 8b). These encompass (i) genes that were shown to be upregulated (*PIM-1*) or repressed (*CEBPA, RGS2* and *PU.1*) by the FLT3-ITD signalling pathway[23,24] (Figure 8a); and (ii) genes that participate in the signature of the *FLT3-ITD* mutation, predicting an adverse clinical outcome in patients presenting a combination of elevated (genes listed Figure 8b, left panel), and reduced (*PDE4A, GNG2*) transcript levels.[25] The comparison of the RNA abundance between CN-AML patients with wild type or bi-allelic mutations of *CEBPA* revealed that most genes were differently expressed in the two groups. Associating with *CEBPA* mutations, variations in *PIM-2* and *CEBPA* expression were inversed to their response to FLT3-ITD signalling (Figure 8a). Strikingly, the *FLT3-ITD* molecular signature,

as determined against the wild-type receptor cases,[25] was largely inverted in CN-AML patients with *CEBPA* bi-allelic mutations when compared with their wild-type counterparts (Figure 8b).

DISCUSSION

Using a murine myeloid leukaemia model and bone marrow cells from AML patients, our study highlights the role of the transcription factors MYB and C/EBPα in the regulation of *FLT3* expression in the leukaemia context.

The cooperative activities of MYB and C/EBPα in activating the promoter of myeloid genes have been previously reported.[26,27] However, *FLT3* constitutes a target of particular significance for both proteins in the context of AML. Having a determinant role in the maintenance and function of the haematopoietic stem cell compartment,[28] MYB activity also associates with a broad spectrum of haematological malignancies, including CML, T-ALL and AML.[29-31] Notably, MYB was reported to have a critical role for the transforming potential of the AML-inducing fusion protein MLL-ENL.[31] However, its function and target genes in the leukaemia context remain poorly understood. Here, by identifying the *FLT3* receptor gene as a direct target of MYB in AML cells, our study places MYB activity upstream to that of FLT3 and thus gives a first indication as to how the proto-oncogene *MYB* may influence the proliferation and survival of AML cells.

Our work suggests that, together with HOXA9 and MEIS1, MYB and C/EBPα are important elements of the combinatorial binding of leukaemia-related transcription factors that regulate *FLT3* expression. The role of HOXA9 and MEIS1 in *FLT3* regulation, and their recruitment to its promoter, had been previously reported.[18] In addition, the recent study of Huang *et al.*[17] considered the existence of enhanceosomes containing these proteins and hinted at the wider relevance of these types of complexes in the regulation of myeloid and leukaemia-related target genes. By analysing the over representation of conserved transcription factor binding sites in the vicinity of HoxA9 and Meis1 binding locations, the authors identified both C/EBPα and MYB as candidate components of these enhanceosomes and suggested a contribution of PU.1. This member of the ETS family of transcription factors was shown to regulate *FLT3* expression in dendritic cells[32] and to cooperate with C/EBPα and MYB in regulating the transcription of the neutrophil elastase gene.[26] Thus, one may expect that a similar collaborative mechanism may underlie the regulation of *FLT3* expression. However, we found no evidence of PU.1 binding to the *Flt3* locus in the CN-AML murine model or any indication of a relation between *PU.1* and *FLT3* expression using the human AML micro-array data. Therefore, in contrast with HOXA9, MEIS1, MYB and C/EBPα, PU.1 does not appear to be a crucial regulator of *FLT3* expression in the context of CN-AML cells.

The fact that C/EBPα participates in *FLT3* regulation is probably the most clinically relevant finding of the present study, as it constitutes a direct link between two major molecular prognostic markers for AML. The individual importance of both *FLT3* and *CEBPA* status for AML prognosis are well documented.[33,34] So far, a few hints of a connection between these molecular markers have been given by recent studies showing that

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the favourable outcome associated with C/EBPα bi-allelic mutations was cancelled out by the presence of *FLT3-ITD* mutations,[6,14] that FLT3-ITD signalling inhibits C/EBPα-differentiating function by promoting its phosphorylation,[35] and that C/EBPα with a *C*-terminal mutation collaborates with FLT3-ITD in inducing AML.[7] Such collaborations may rely on the ability of FLT3 signalling to support myeloid commitment of the expanding *CEBPA* mutated cells.[6] Our analysis of profiling arrays from AML patients with differing *CEBPA* status links *FLT3* expression and C/EBPα activity. We find that *CEBPA* bi-allelic mutations associate with lower levels of *FLT3* transcript in AML patients. In a bid to assess the influence of C/EBPα mutated forms on *Flt3* expression, we introduced N- and *C*-terminal mutants, singly or in combination, in the FMH9 cell-line (Supplementary Figure S3). This approach failed to induce statistically significant changes in *Flt3* expression, possibly due to the presence of the wild-type form. Similarly, analysis of the profiling arrays did not reveal significant differences in *FLT3* expression between patients with-wild type or mono-allelic CEBPA mutations. Conversely, global impairment of C/EBPα activity through shRNA silencing resulted in *Flt3* downregulation. Our cell-line based results also suggested that a tight control on C/EBPα expression at the cellular level might prevent any sizeable over-expression. Arguing for the need to maintain such stability, small increases in *Cebpa* also resulted in reduced *Flt3* RNA levels. The explanation for this could lie within a delicate equilibrium between some constituents of the leukaemic transcriptional network. Alternatively, the apparent decrease in *Flt3* expression may reflect a change in the cellular context. In fact, small changes in C/EBPα expression may induce granulocytic differentiation of transformed cells, as previously demonstrated in U937 cells.[36] In the context of the disease, it is possible that the lower levels of *FLT3* in cells from patients with *CEBPA* bi-allellic mutations may prevent auto-phosphorylation of the FLT3 receptor that occurs when the *FLT3* RNA level exceeds 200 000 copies/mg RNA.[15] This hypothesis would be consistent with the findings that *CEBPA* bi-allelic mutations,[13,37] or more generally loss of DNA-binding activity,[38] associate with a positive prognosis. As this benefit is lost in the presence of FLT3 activating mutations, it would be very interesting to test this theory by assessing whether auto-activation of the FLT3 receptor is less frequent in AML with *CEBPA* bi-allelic mutations and how this may contribute to the favourable prognosis associated with this status. Here, we analysed the expression level of genes that constitute the molecular signature of CN-AML with *FLT3-ITD* mutation.[25] With reference to CN-AML with wild-type *CEBPA*, we found that patients with *CEBPA* bi-allelic mutations present an inverse expression pattern to that of *FLT3-ITD* patients. Being regulated in an opposite manner by the wild-type and mutated receptors signalling cascades,[23,24] *CEBPA* itself is a notable downstream target. Its elevated expression in patients with bi-allelic mutations further undermines the idea of a FLT3-ITD-related signalling cascade in these patients. Taken together, our results support the hypothesis that the presence of bi-allelic *CEBPA* mutations may reduce FLT3-mediated leukemogenic signals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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REFERENCES

1. Gregory TK, Wald D, Chen Y, Vermaat JM, Xiong Y, Tse W. Molecular prognostic markers for adult acute myeloid leukemia with normal cytogenetics. J Hematol Oncol. 2009; 2:23. [PubMed: 19490647]
2. Drexler HG. Expression of FLT3 receptor and response to FLT3 ligand by leukemic cells. Leukemia. 1996; 10:588–599. [PubMed: 8618433]
3. Rosnet O, Buhring HJ, deLapeyriere O, Beslu N, Lavagna C, Marchetto S, et al. Expression and signal transduction of the FLT3 tyrosine kinase receptor. Acta Haematol. 1996; 95:218–223. [PubMed: 8677746]
4. Kindler T, Lipka DB, Fischer T. FLT3 as a therapeutic target in AML: still challenging after all these years. Blood. 2010; 116:5089–5102. [PubMed: 20705759]
5. Kelly LM, Liu Q, Kutok JL, Williams IR, Boulton CL, Gilliland DG. FLT3 internal tandem duplication mutations associated with human acute myeloid leukemias induce myeloproliferative disease in a murine bone marrow transplant model. Blood. 2002; 99:310–318. [PubMed: 11756186]
6. Reckzeh K, Bereshchenko O, Mead A, Rehn M, Kharazi S, Jacobsen SE, et al. Molecular and cellular effects of oncogene cooperation in a genetically accurate AML mouse model. Leukemia. 2012; 26:1527–1536. [PubMed: 22318449]
7. Kato N, Kitaura J, Doki N, Komeno Y, Watanabe-Okochi N, Togami K, et al. Two types of C/EBPalpha mutations play distinct but collaborative roles in leukemo-genesis: lessons from clinical data and BMT models. Blood. 2011; 117:221–233. [PubMed: 20884804]
8. Zhang DE, Zhang P, Wang ND, Hetherington CJ, Darlington GJ, Tenen DG. Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha-deficient mice. Proc Natl Acad Sci USA. 1997; 94:569–574. [PubMed: 9012825]
9. Hasemann MS, Damgaard I, Schuster MB, Theilgaard-Monch K, Sorensen AB, Mrsic A, et al. Mutation of C/EBPalpha predisposes to the development of myeloid leukemia in a retroviral insertional mutagenesis screen. Blood. 2008; 111:4309–4321. [PubMed: 18212247]
10. Leroy H, Roumier C, Huyghe P, Biggio V, Fenaux P, Preudhomme C. CEBPA point mutations in hematological malignancies. Leukemia. 2005; 19:329–334. [PubMed: 15674366]
11. Nerlov C. C/EBPalpha mutations in acute myeloid leukaemias. Nat Rev Cancer. 2004; 4:394–400. [PubMed: 15122210]
12. Pabst T, Mueller BU. Transcriptional dysregulation during myeloid transformation in AML. Oncogene. 2007; 26:6829–6837. [PubMed: 17934489]
13. Dufour A, Schneider F, Metzeler KH, Hoster E, Schneider S, Zellmeier E, et al. Acute myeloid leukemia with biallelic CEBPA gene mutations and normal karyotype represents a distinct genetic entity associated with a favorable clinical outcome. J Clin Oncol. 2010; 28:570–577. [PubMed: 20038735]
14. Renneville A, Boissel N, Gachard N, Naguib D, Bastard C, de Botton S, et al. The favorable impact of CEBPA mutations in patients with acute myeloid leukemia is only observed in the absence of associated cytogenetic abnormalities and FLT3 internal duplication. Blood. 2009; 113:5090–5093. [PubMed: 19289855]
15. Ozeki K, Kiyoi H, Hirose Y, Iwai M, Ninomiya M, Kodera Y, et al. Biologic and clinical significance of the FLT3 transcript level in acute myeloid leukemia. Blood. 2004; 103:1901–1908. [PubMed: 14604973]

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16. Dicker F, Haferlach C, Kern W, Haferlach T, Schnittger S. Trisomy 13 is strongly associated with AML1/RUNX1 mutations and increased FLT3 expression in acute myeloid leukemia. Blood. 2007; 110:1308–1316. [PubMed: 17485549]
17. Huang Y, Sitwala K, Bronstein J, Sanders D, Dandekar M, Collins C, et al. Identification and characterization of Hoxa9 binding sites in hematopoietic cells. Blood. 2012; 119:388–398. [PubMed: 22072553]
18. Wang GG, Pasillas MP, Kamps MP. Persistent transactivation by meis1 replaces hox function in myeloid leukemogenesis models: evidence for co-occupancy of meis1-pbx and hox-pbx complexes on promoters of leukemia-associated genes. Mol Cell Biol. 2006; 26:3902–3916. [PubMed: 16648484]
19. Lorvellec M, Dumon S, Maya-Mendoza A, Jackson D, Frampton J, Garcia P. B-Myb is critical for proper DNA duplication during an unperturbed S phase in mouse embryonic stem cells. Stem Cells. 2010; 28:1751–1759. [PubMed: 20715180]
20. Harr B, Schlotterer C. Comparison of algorithms for the analysis of Affymetrix microarray data as evaluated by co-expression of genes in known operons. Nucleic Acids Res. 2006; 34:e8. [PubMed: 16432259]
21. Kroon E, Krosl J, Thorsteinsdottir U, Baban S, Buchberg AM, Sauvageau G. Hoxa9 transforms primary bone marrow cells through specific collaboration with Meis1a but not Pbx1b. EMBO J. 1998; 17:3714–3725. [PubMed: 9649441]
22. Thorsteinsdottir U, Kroon E, Jerome L, Blasi F, Sauvageau G. Defining roles for HOX and MEIS1 genes in induction of acute myeloid leukemia. Mol Cell Biol. 2001; 21:224–234. [PubMed: 11113197]
23. Mizuki M, Schwable J, Steur C, Choudhary C, Agrawal S, Sargin B, et al. Suppression of myeloid transcription factors and induction of STAT response genes by AML-specific Flt3 mutations. Blood. 2003; 101:3164–3173. [PubMed: 12468433]
24. Schwable J, Choudhary C, Thiede C, Tickenbrock L, Sargin B, Steur C, et al. RGS2 is an important target gene of Flt3-ITD mutations in AML and functions in myeloid differentiation and leukemic transformation. Blood. 2005; 105:2107–2114. [PubMed: 15536149]
25. Bullinger L, Dohner K, Kranz R, Stirner C, Frohling S, Scholl C, et al. An FLT3 gene-expression signature predicts clinical outcome in normal karyotype AML. Blood. 2008; 111:4490–4495. [PubMed: 18309032]
26. Oelgeschlager M, Nuchprayoon I, Luscher B, Friedman AD. C/EBP, c-Myb, and PU.1 cooperate to regulate the neutrophil elastase promoter. Mol Cell Biol. 1996; 16:4717–4725. [PubMed: 8756629]
27. Burk O, Mink S, Ringwald M, Klempnauer KH. Synergistic activation of the chicken mim-1 gene by v-myb and C/EBP transcription factors. EMBO J. 1993; 12:2027–2038. [PubMed: 8491193]
28. Lieu YK, Reddy EP. Conditional c-myb knockout in adult hematopoietic stem cells leads to loss of self-renewal due to impaired proliferation and accelerated differentiation. Proc Natl Acad Sci USA. 2009; 106:21689–21694. [PubMed: 19955420]
29. Lidonnici MR, Corradini F, Waldron T, Bender TP, Calabretta B. Requirement of c-Myb for p210(BCR/ABL)-dependent transformation of hematopoietic progenitors and leukemogenesis. Blood. 2008; 111:4771–4779. [PubMed: 18227349]
30. Clappier E, Cuccuini W, Kalota A, Crinquette A, Cayuela JM, Dik WA, et al. The C-MYB locus is involved in chromosomal translocation and genomic duplications in human T-cell acute leukemia (T-ALL), the translocation defining a new T-ALL subtype in very young children. Blood. 2007; 110:1251–1261. [PubMed: 17452517]
31. Hess JL, Bittner CB, Zeisig DT, Bach C, Fuchs U, Borkhardt A, et al. c-Myb is an essential downstream target for homeobox-mediated transformation of hematopoietic cells. Blood. 2006; 108:297–304. [PubMed: 16507773]
32. Carotta S, Dakic A, D'Amico A, Pang SH, Greig KT, Nutt SL, et al. The transcription factor PU.1 controls dendritic cell development and Flt3 cytokine receptor expression in a dose-dependent manner. Immunity. 2010; 32:628–641. [PubMed: 20510871]
33. Meshinchi S, Appelbaum FR. Structural and functional alterations of FLT3 in acute myeloid leukemia. Clin Cancer Res. 2009; 15:4263–4269. [PubMed: 19549778]

34. Pabst T, Mueller BU. Complexity of CEBPA dysregulation in human acute myeloid leukemia. Clin Cancer Res. 2009; 15:5303–5307. [PubMed: 19706798]
35. Radomska HS, Alberich-Jorda M, Will B, Gonzalez D, Delwel R, Tenen DG. Targeting CDK1 promotes FLT3-activated acute myeloid leukemia differentiation through C/EBPalpha. J Clin Invest. 2012; 122:2955–2966. [PubMed: 22797303]
36. Radomska HS, Huettner CS, Zhang P, Cheng T, Scadden DT, Tenen DG. CCAAT/enhancer binding protein alpha is a regulatory switch sufficient for induction of granulocytic development from bipotential myeloid progenitors. Mol Cell Biol. 1998; 18:4301–4314. [PubMed: 9632814]
37. Wouters BJ, Lowenberg B, Erpelinck-Verschueren CA, van Putten WL, Valk PJ, Delwel R. Double CEBPA mutations, but not single CEBPA mutations, define a subgroup of acute myeloid leukemia with a distinctive gene expression profile that is uniquely associated with a favorable outcome. Blood. 2009; 113:3088–3091. [PubMed: 19171880]
38. Fos J, Pabst T, Petkovic V, Ratschiller D, Mueller BU. Deficient CEBPA DNA binding function in normal karyotype AML patients is associated with favorable prognosis. Blood. 2011; 117:4881–4884. [PubMed: 21389317]

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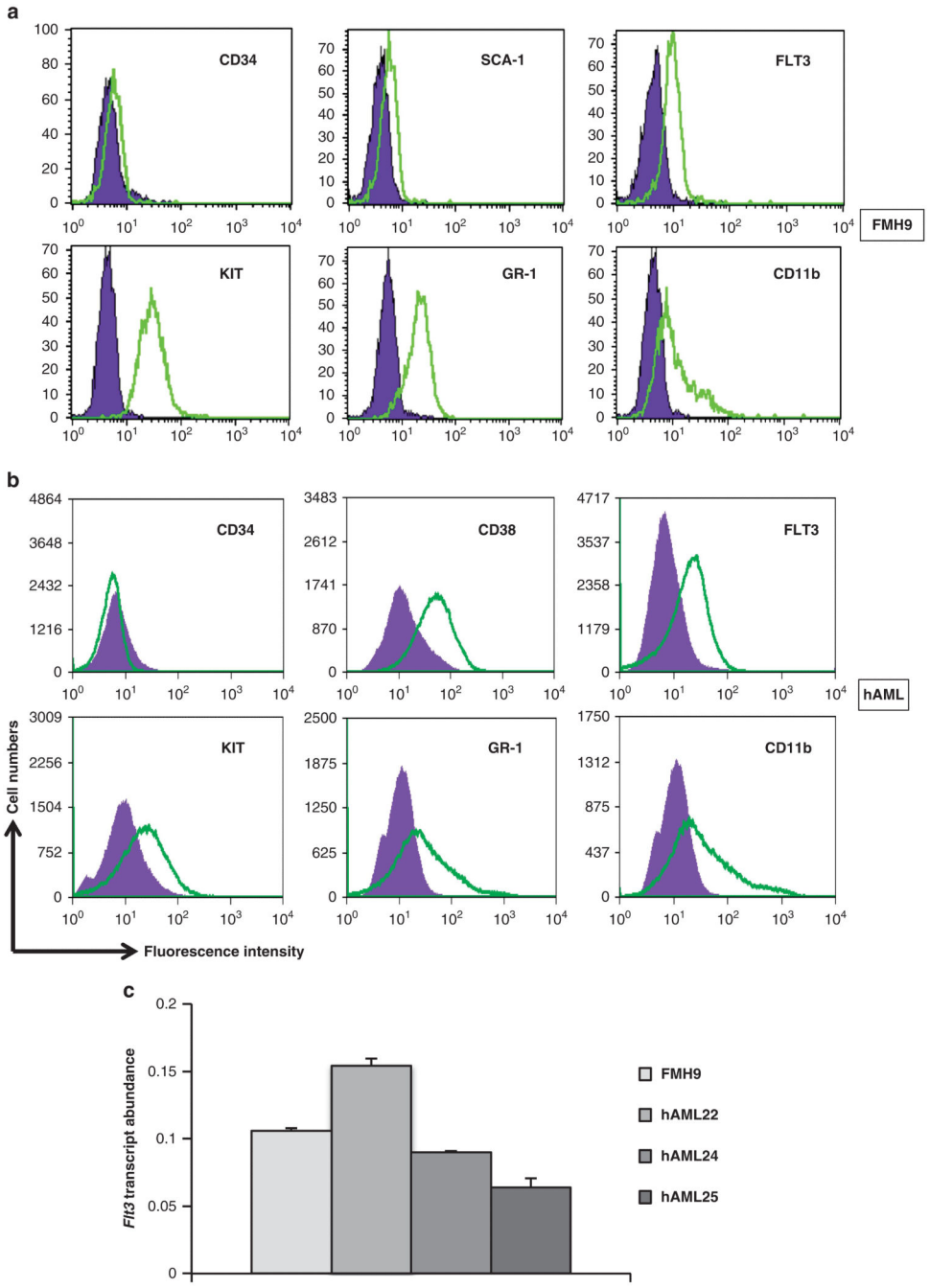

Figure 1. FMH9 cells and AML cells flow cytometric characterisation.

(**a**) (**b**) Staining with labelled antibodies against stem cell-related antigens (KIT, SCA-1, CD34, FLT3) and lineage specific markers (GR-1, CD11b) are overlaid against matched isotype controls. (**c**) *FLT3* mRNA quantification by quantitative-PCR, normalised against the 18S house keeping gene results.

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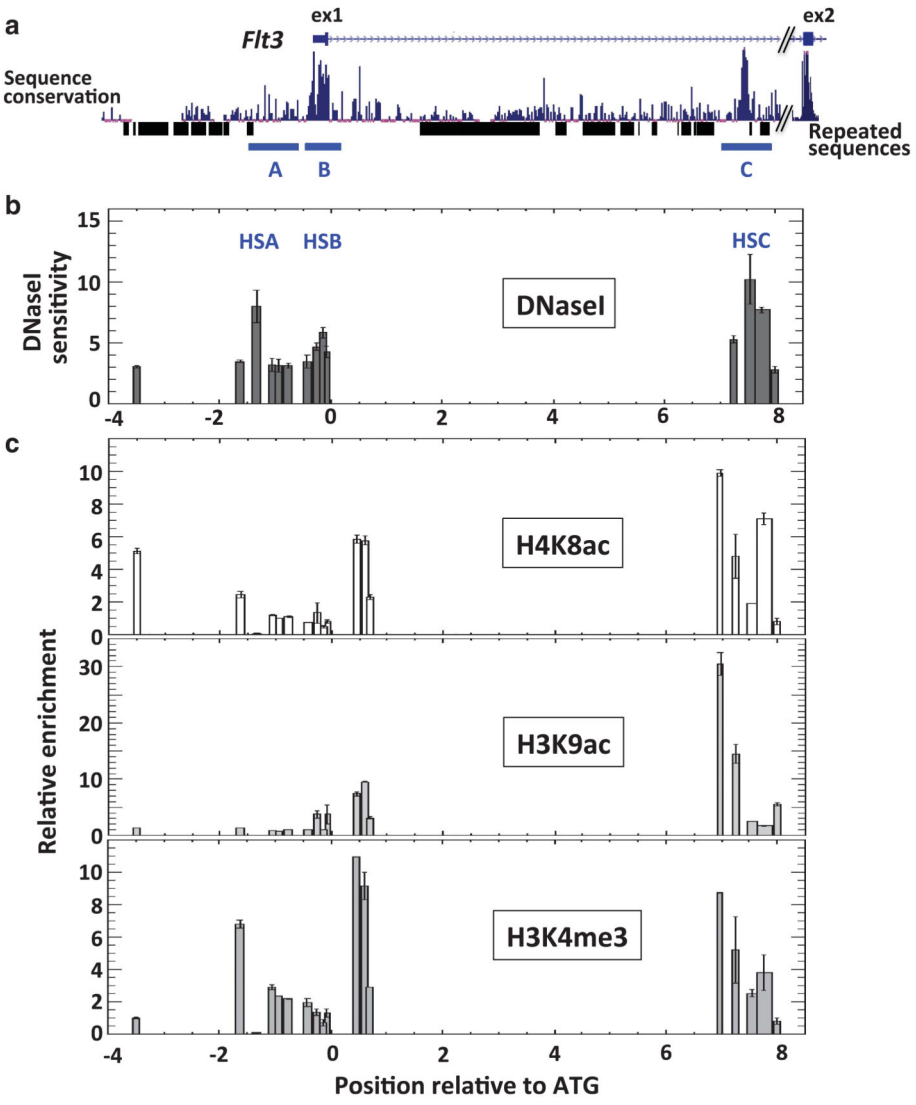

Figure 2. *Flt3* regulatory modules.

(**a**) Schematic representation of the *Flt3* promoter and first intron (filled boxes indicate exon1 and 2). The level of sequence conservation between seven mammalian species and the position of repeated sequences are indicated below. The global illustration is aligned to the lower (**b**) and bottom (**c**) plots. (**b**) DNaseI HSS mapping at the *Flt3* locus. Ratios between quantitative-PCR results from partially digested and untreated samples ($2^{-\Delta Ct}$) reflect the extent of nuclease sensitivity across regions covered by PCR amplicons. (c) Histone mark distribution at the *Flt3* locus in the murine FMH9 AML model. Relative enrichments are determined against the control immunoglobulin G X-ChIP material.

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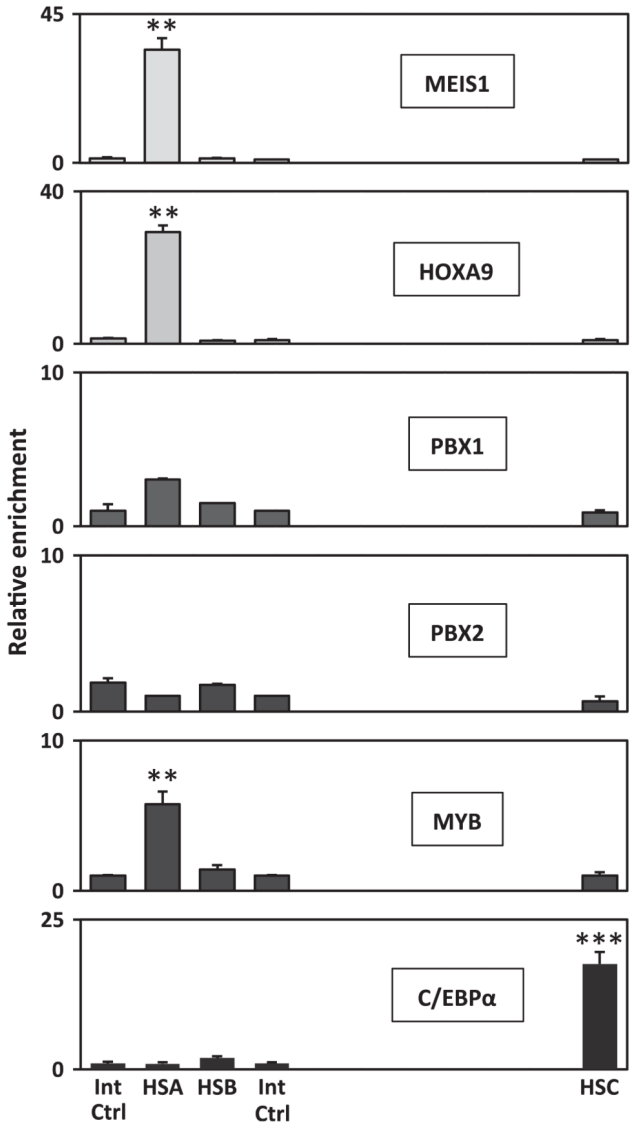

Figure 3. Detection of transcription factor binding at the sites of nuclease hypersensitivity.

Relative enrichments were determined against the immunoglobulin G control material by quantitative-PCR at the location of the hypersensitive regions (HSA to C at and – 1.45, – 0.15 + 7.5 kb relative to the *Flt3* initiating codon) and normalised against two internal control regions (at 3.5 and – 0.27 kb from the ATG). Error bars represent the s.e. of – the mean. All plots are representative of a minimum of three independent experiments used to determine the two-tailed *P*-value by paired *t*-test: ***00.001 and **00.01.

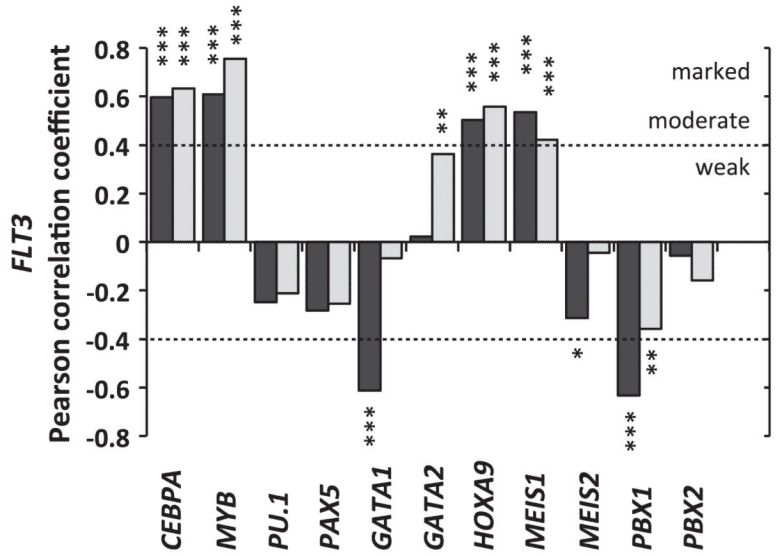

Figure 4. Expression codependency between FLT3 and its potential transcriptional regulators in CN-AML measured by the Pearson correlation test.

The correlation coefficients were determined using log expression values from two sets of arrays summing 46 and 58 CN-AML patients without CEBPA mutations. Statistical significance ***<0.001 **<0.01 and *<0.05.

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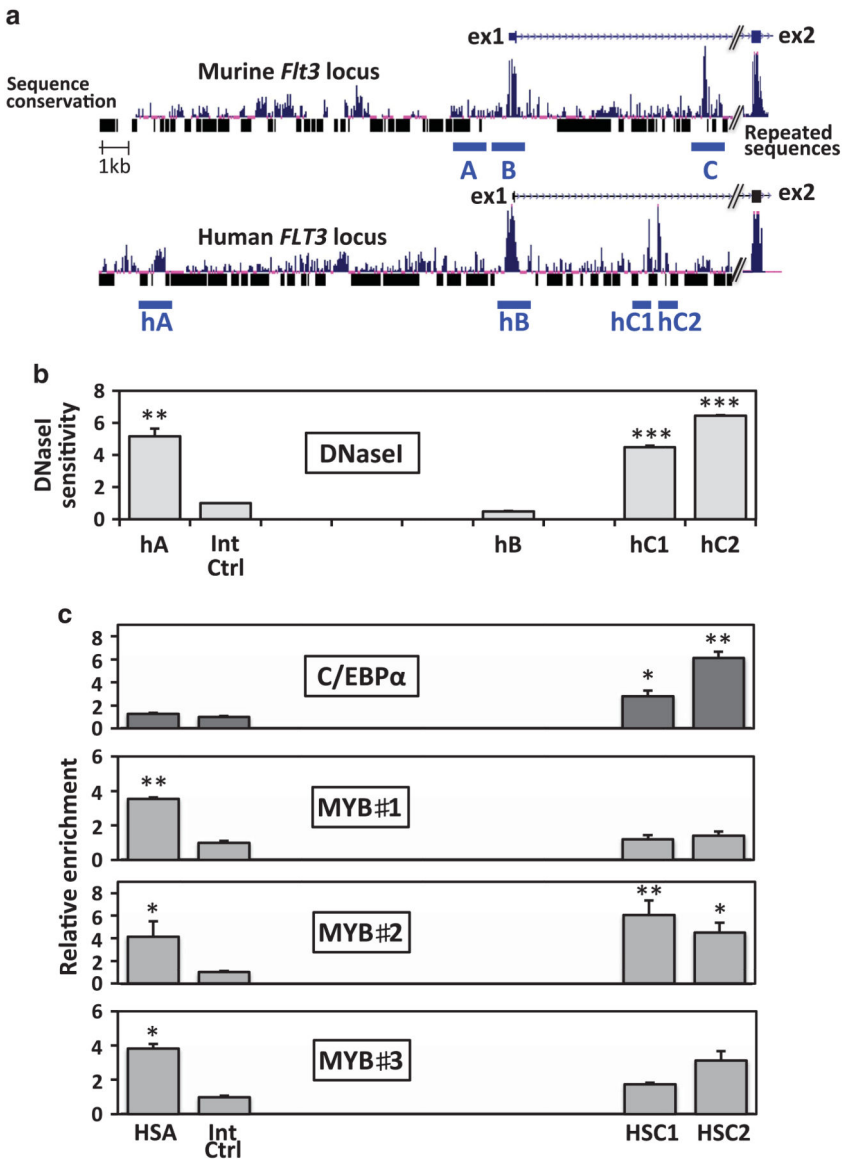

Figure 5. C/EBPα and MYB bind the *FLT3* locus in human AML cells.

(**a**) Schematic comparison of the murine and human *FLT3* promoter and first intron (filled boxes represent exons) with indication of sequence conservation and repeated sequence locations. (**b**) DNaseI hypersensitivity mapping at the human *FLT3* locus in CN-AML patients. Plotted for the human sequence orthologs of the murine HSA, HSB, HSC and a control region, ratios between quantitative-PCR results from partially digested and untreated samples are representative of four experiments. (**c**) Detection of MYB and C/EBPα *in vivo* binding at the sites of nuclease hypersensitivity, located at – 15.3 kb (hA), –0.37 kb (hB) and + 6.2 kb (hC1/C2) from the human *FLT3* initiating codon. Relative enrichments were determined against the immunoglobulin G control material and normalised against the control region (at –6.95 kb from the ATG). Error bars represent the s.e. of the mean. C/EBPα ChIP plots are representative of three independent experiments used to determine the two-tailed P-value by paired *t*-test. MYB ChIP plots represent patient-specific experiments

for which the *P*-values were calculated using replicate measurements. P-value indications ***<0.001, **<0.01 and *<0.05.

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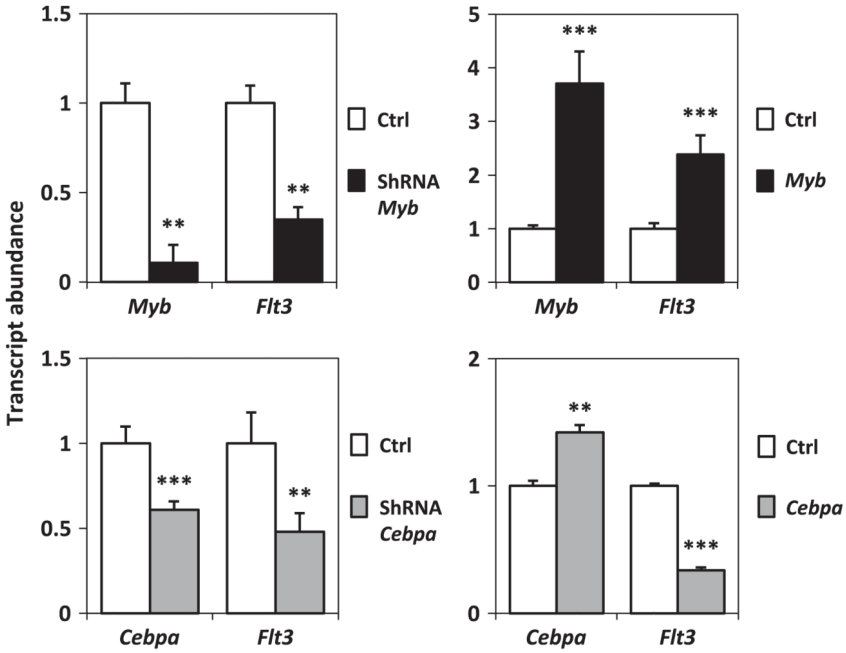

Figure 6. Silencing and ectopic expression of MYB and C/EBPα in FMH9 cells.

Transcript abundances were determined by quantitative-PCR in cells transfected with shRNA and expression vectors, normalised against the *Gapdh* house keeping gene PCR result and compared with the correspondent empty vector controls. Results are representative of three or four independent experiments. Statistical significance ***<0.001 and **<0.01.

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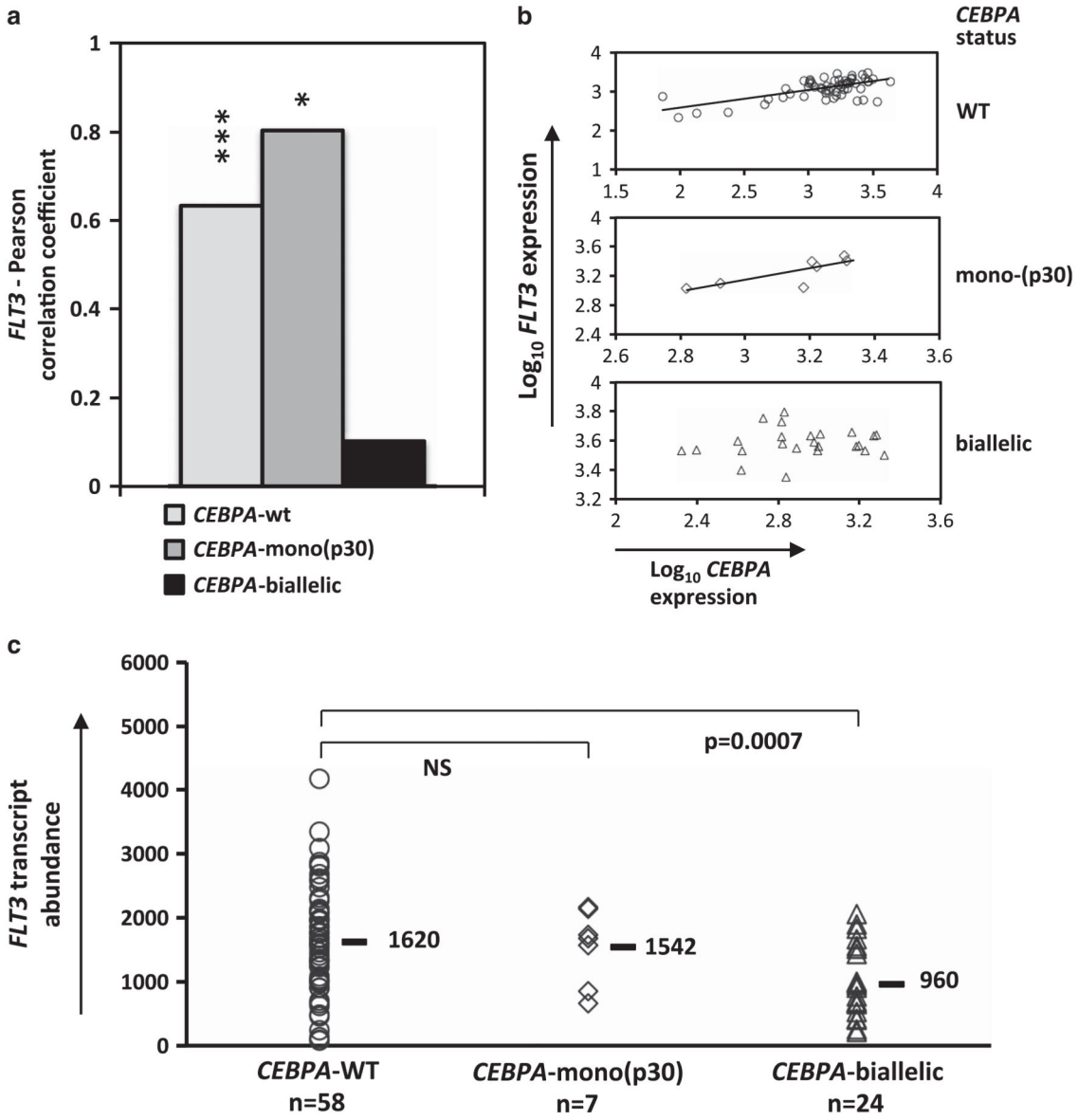

Figure 7.

(**a**) Pearson correlation test between *FLT3* and *CEBPA* transcript levels applied to CEBPAWT, CEBPASM and CEBPADM patient arrays. Statistical significance: ***<0.001 and *<0.05. (**b**) Scatter plots and trend line fitting between *FLT3* and *CEBPA* expression in the different subgroups (c) FLT3 mRNA levels in AML patients with wild-type mono- or bi-allelic *CEBPA* mutations. Median values are indicated. Differential statistical significances are indicated through *P*-values or non-significance sign NS.

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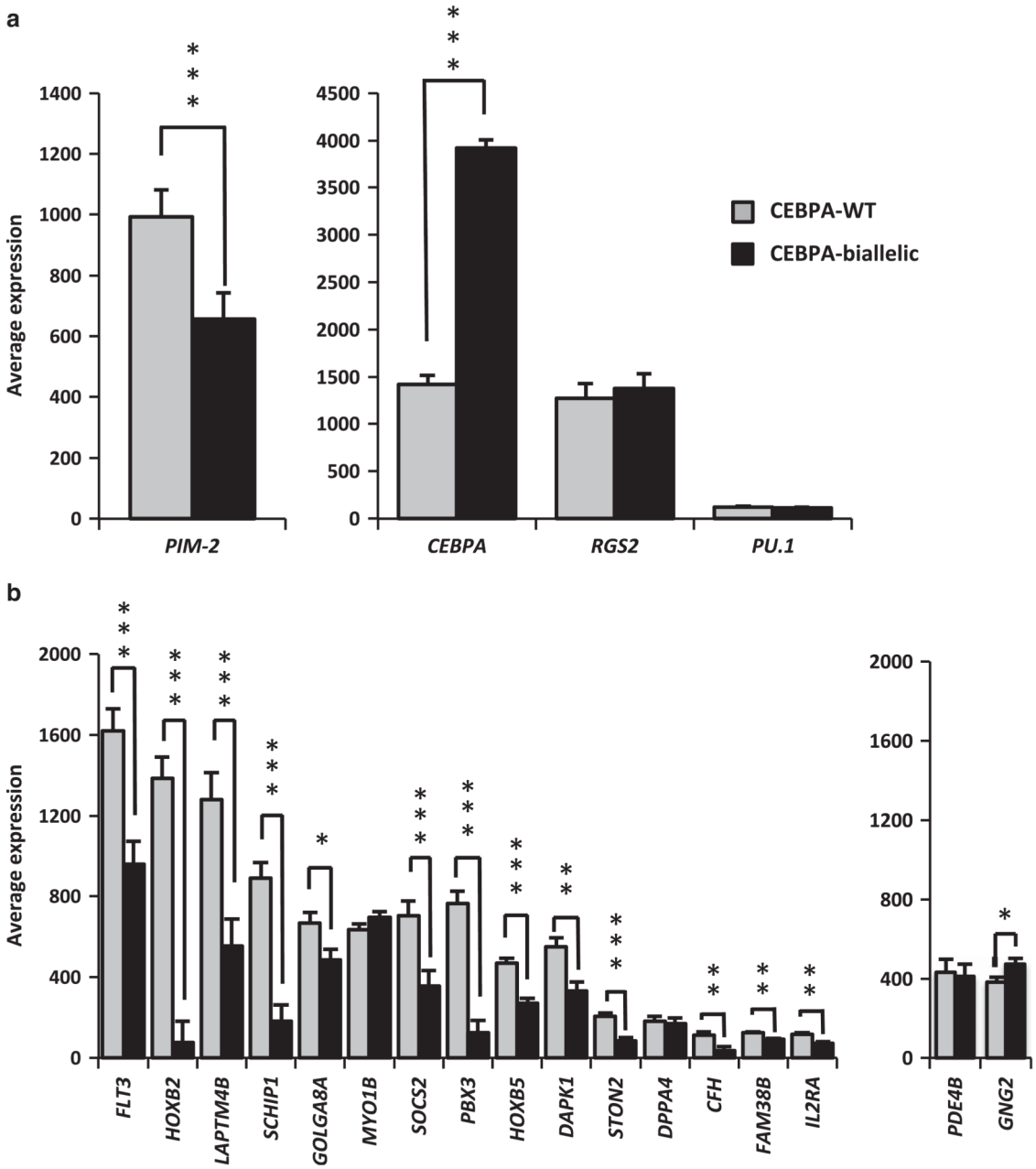

Figure 8. Patients with wild-type and bi-allelic CEBPA mutations differentially express genes that are part of the signature of the constitutively activated mutant receptor FLT3-ITD.

Bars represent the average expression in AML patients with wild-type and bi-allelic *CEBPA* mutations and error bars depict the s.e. of the mean. Tested genes were previously shown to be up-(left panel) or downregulated (right panel) in response to FLT3-ITD signalling (**a**) or are elements of the FLT3-ITD signature in CN-AML (**b**). Statistical significance: ***<0.001, **<0.01 and *<0.05.