

## MYELOID NEOPLASIA

Chemo-genomic interrogation of *CEBPA* mutated AML reveals recurrent *CSF3R* mutations and subgroup sensitivity to JAK inhibitors

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## Key Points

- *CSF3R* was the most frequently mutated gene identified in this *CEBPA*<sup>bi</sup> AML cohort analyzed by next-generation sequencing.
- *CEBPA*<sup>bi</sup> AML that have a characteristic transcriptomic profile are more sensitive to JAK inhibitors than *CEBPA*<sup>wt</sup> AML.

In this study, we analyzed RNA-sequencing data of 14 samples characterized by biallelic *CEBPA* (*CEBPA*<sup>bi</sup>) mutations included in the LeuceGene collection of 415 primary acute myeloid leukemia (AML) specimens, and describe for the first time high frequency recurrent mutations in the granulocyte colony-stimulating factor receptor gene *CSF3R*, which signals through JAK-STAT proteins. Chemical interrogation of these primary human specimens revealed a uniform and specific sensitivity to all JAK inhibitors tested irrespective of their *CSF3R* mutation status, indicating a general sensitization of JAK-STAT signaling in this leukemia subset. Altogether, these results identified the co-occurrence of mutations in *CSF3R* and *CEBPA* in a well-defined AML subset, which uniformly responds to JAK inhibitors and paves the way to personalized clinical trials for this disease. (*Blood*. 2016;127(24):3054-3061)

*CEBPA* (CCAAT/enhancer binding protein  $\alpha$ ) encodes a 42-kDa transcription factor essential for differentiation of myeloid progenitor cells. *CEBPA* regulates expression of the granulocyte colony-stimulating factor receptor (*CSF3R*) gene, which plays a prominent role in granulocyte differentiation.<sup>1</sup> Homozygous deletion of *CEBPA* in mouse hematopoietic cells leads to a selective loss of *CSF3R* expression and results in a complete block of neutrophil differentiation.<sup>1,2</sup> Acquired mutations in *CSF3R* are present in a majority of chronic myeloid leukemias and atypical (*BCR-ABL1*-negative) chronic myeloid leukemias,<sup>3</sup> which are neoplasms affecting the granulocytic lineage. *CSF3R* mutations comprise either the membrane-proximal missense mutations or C-terminal truncating mutations proposed to lead to ligand independence and ligand hypersensitivity, respectively.<sup>4</sup> *CSF3R* mutations have also been described in patients with congenital neutropenia treated with granulocyte colony-stimulating factor therapy upon acute myeloid leukemia (AML) transformation,<sup>5,6</sup> but they have only been reported in <1% to 2% of AML.<sup>3,7,8</sup>

Two major categories of collaborating *CEBPA* mutations have been described in human AML: (1) frameshift (FS) insertions or deletions affecting the N-terminal region resulting in a loss of the 42 kDa protein and overexpression of a shorter 30 kDa *CEBPA* protein proposed to

exhibit a dominant negative activity,<sup>9</sup> and (2) in frame mutations in the C-terminal region that alter the basic leucine zipper domain.<sup>10</sup> *CEBPA*-mutated AML carrying mutations in both alleles (*CEBPA* biallelic [*CEBPA*<sup>bi</sup>] AML) represent a distinct subgroup characterized by a normal karyotype (NK) and favorable prognosis.<sup>11,12</sup> In most specimens, a combination of N-terminal FS and C-terminal in frame mutations are observed, hereafter called typical *CEBPA*<sup>bi</sup> AML. Other combinations of *CEBPA*<sup>bi</sup> mutations, hereafter called atypical *CEBPA*<sup>bi</sup> AML, were also described.<sup>13,14</sup>

Gene expression studies have shown that *CEBPA*<sup>bi</sup> samples, but not specimens with *CEBPA* monoallelic mutations, have a distinctive gene expression profile (GEP),<sup>15-18</sup> and analyses of small series indicated a possibility that this profile is shared by some atypical *CEBPA*<sup>bi</sup> AML.<sup>15</sup> Mutations in genes such as *GATA2*, *WT1*, and *TET2* have been described thus far in *CEBPA*<sup>bi</sup> specimens,<sup>19-22</sup> including 6 specimens reported in The Cancer Genome Atlas cohort.<sup>7</sup>

We previously used comparative transcriptomic approaches to report the mutational and transcriptional landscapes of *MLL*,<sup>23</sup> *EVII*,<sup>24</sup> *NUP98-NSD1*,<sup>25</sup> and *CBF*<sup>26</sup> AML subgroups included in the LeuceGene cohort, and also demonstrated that chemical interrogation of a mutation could identify new therapeutic targets in AML.<sup>23</sup>

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We hereby describe RNA-sequencing analysis of the 14 *CEBPA*<sup>bi</sup> AML specimens included in our collection, and report new activating signaling mutations in this disease, which revealed the sensitivity of this subgroup to compounds that specifically affect the JAK-STAT signaling pathway.

## Methods

### Human leukemia and normal samples

The Leucegene project is an initiative approved by the Research Ethics Boards of the Université de Montréal and Maisonneuve-Rosemont Hospital. As part of this project, RNA sequencing of 415 primary AML specimens from various cytogenetic groups was performed, including 110 samples that were also characterized by exome sequencing, as previously described.<sup>23</sup> All leukemia samples and paired normal DNA specimens were collected and characterized by the Quebec Leukemia Cell Bank (BCLQ). Normal bone marrow (BM) samples were obtained from the BCLQ and from Lonza, and cord blood from Héma-Québec.<sup>23</sup>

### Next-generation sequencing (NGS) and mutation validations

Sequencing was performed as previously described.<sup>23</sup> Sequence data were mapped to the reference genome hg19 according to RefSeq annotations (University of California Santa Cruz; April 16th, 2014). Variants were all identified using CASAVA 1.8.2 or km (<https://bitbucket.org/iric-soft/km>) approaches according to the previously reported pipeline.<sup>24,25</sup> All variants present in 80 genes mutated in myeloid cancers or in acute leukemias were investigated (see supplemental Table 1, available on the *Blood* Web site). Acquired or germ line origin of these variants not present in the Catalogue of Somatic Mutations in Cancer database were all confirmed by Sanger sequencing of nontumoral DNA from mouth swabs or saliva. Other genes with recurrent variants (ie, in 3 or more *CEBPA*<sup>bi</sup> samples) were also analyzed in nontumoral DNA. Samples with *CEBPA* variant coverage <10× were confirmed by tumor DNA Sanger sequencing. Samples from the *CEBPA*<sup>bi</sup> group with no detectable *WT1* mutations were also analyzed by tumor complementary DNA sequencing of *WT1* exons 6-10 (based on NM\_001198551). *NRAS*, *KRAS*, and *PTPN11* mutations were detected at a variant allele frequency (VAF) ≥5%, and *FLT3*-internal tandem duplications with a VAF ≥10% were reported.

For the analysis of variants in *JAK*-related genes, all variants in coding regions of genes in supplemental Table 2 identified using CASAVA 1.8.2 (≥8 variant reads and ≥20× coverage) are reported, after filtering out variants also present in normal samples (n = 67 sequenced populations) and those present in samples resistant to ruxolitinib (IC<sub>50</sub> >100 nM). For enrichment calculation, frequency of variant in samples sensitive to ruxolitinib (IC<sub>50</sub> <100 nM) was divided by the frequency in normal individuals based on the Single Nucleotide Polymorphism database version 137. The frequency of variants in normal individuals that are not cataloged in the Single Nucleotide Polymorphism database was arbitrarily set at 1/1000. Enrichment was not calculated (NA) for variants present in only 1 sensitive sample.

### Normal myeloid populations

The following populations were sorted from normal BM on a BD Aria II cell sorter using the corresponding panels as denoted here. Promyelocytes: propidium iodide (PI)-/CD34<sup>-</sup>/CD16<sup>-</sup>/CD11b<sup>-</sup>/CD33<sup>+</sup>/CD15<sup>+</sup>; myelocytes: PI-/CD34<sup>-</sup>/CD16<sup>-</sup>/CD11b<sup>+</sup>/CD13<sup>-</sup>/CD15<sup>+</sup>/CD33<sup>+</sup>; metamyelocytes: PI-/CD34<sup>-</sup>/CD16int/CD11b<sup>+</sup>/CD33int/CD15<sup>+</sup>; and band and segmented granulocytes: PI-/CD34<sup>-</sup>/CD16high/CD11b<sup>+</sup>/CD33int/CD13<sup>+</sup>/CD15<sup>+</sup>. Granulocytes were sorted from normal peripheral blood by gating on SSC<sup>++</sup>CD33int cells. Fresh normal BM was purchased from Lonza. The following fluorescence-activated cell sorter antibodies were used: CD33 PE (555450; BD Biosciences), CD34 allophycocyanin (555824; BD Biosciences), CD11b PE-Cy5 (555389; BD Biosciences), CD16 Pacific Blue (558122; BD Biosciences),

CD13 allophycocyanin-Cy7 (301710; BD Biosciences), and CD15 FITC (555401; BD Biosciences).

### Cell culture and chemical screen

Preparation of cell culture from frozen AML mono-nucleated cells and chemical screen were performed as previously described<sup>23,27</sup> using serum-free media supplemented with cytokines, 500 nM SR1 (Alicem) and 500 nM UM729 (Institute for Research in Immunology and Cancer [IRIC]). Compounds were added to seeded cells in serial dilutions (8 dilutions, 1:3, 10 μM down to 4.5 nM) in duplicate wells. The exception was daunorubicin for which dilutions from 1 μM to 0.45 nM were performed. Control wells received dimethyl sulfoxide (0.1%) only. Cell viability was evaluated after 6-day culture using the CellTiterGlo assay (Promega) according to the manufacturer's instructions. Percentage of inhibition for dose-response curves was calculated as 100 - (100 × [mean luminescence (compound)/mean luminescence (dimethyl sulfoxide)]). IC<sub>50</sub> values were calculated using ActivityBase SARview Suite. Dose-response curves were generated using nonlinear regression in GraphPad Prism 4.03. For cases where compounds failed to inhibit AML cell survival/proliferation, IC<sub>50</sub> values were arbitrarily reported at the highest dose tested (10 000 nM).

### Statistical analyses

Fisher's exact test was used in the analysis of contingency tables. Analysis of differential gene expression was performed using the Wilcoxon rank-sum test, and the false discovery rate method was applied for global gene analysis as previously described.<sup>23</sup>

Gene ontology enrichment analysis was performed by computing overlaps with curated gene sets from MSigDB (via a hypergeometric test) using the provided tool on the Broad Institute website. Principal component analyses (PCA) were performed on log<sub>10</sub> transformed reads per kilobase per million (RPKM) values using *prcomp* function in the *stats* package and visualized using *ggbiplot* package in R version 3.1.2. Differences in response to small molecules between genetic groups were evaluated using a Wilcoxon rank-sum test performed on IC<sub>50</sub> values in R version 3.1.2.

## Results and discussion

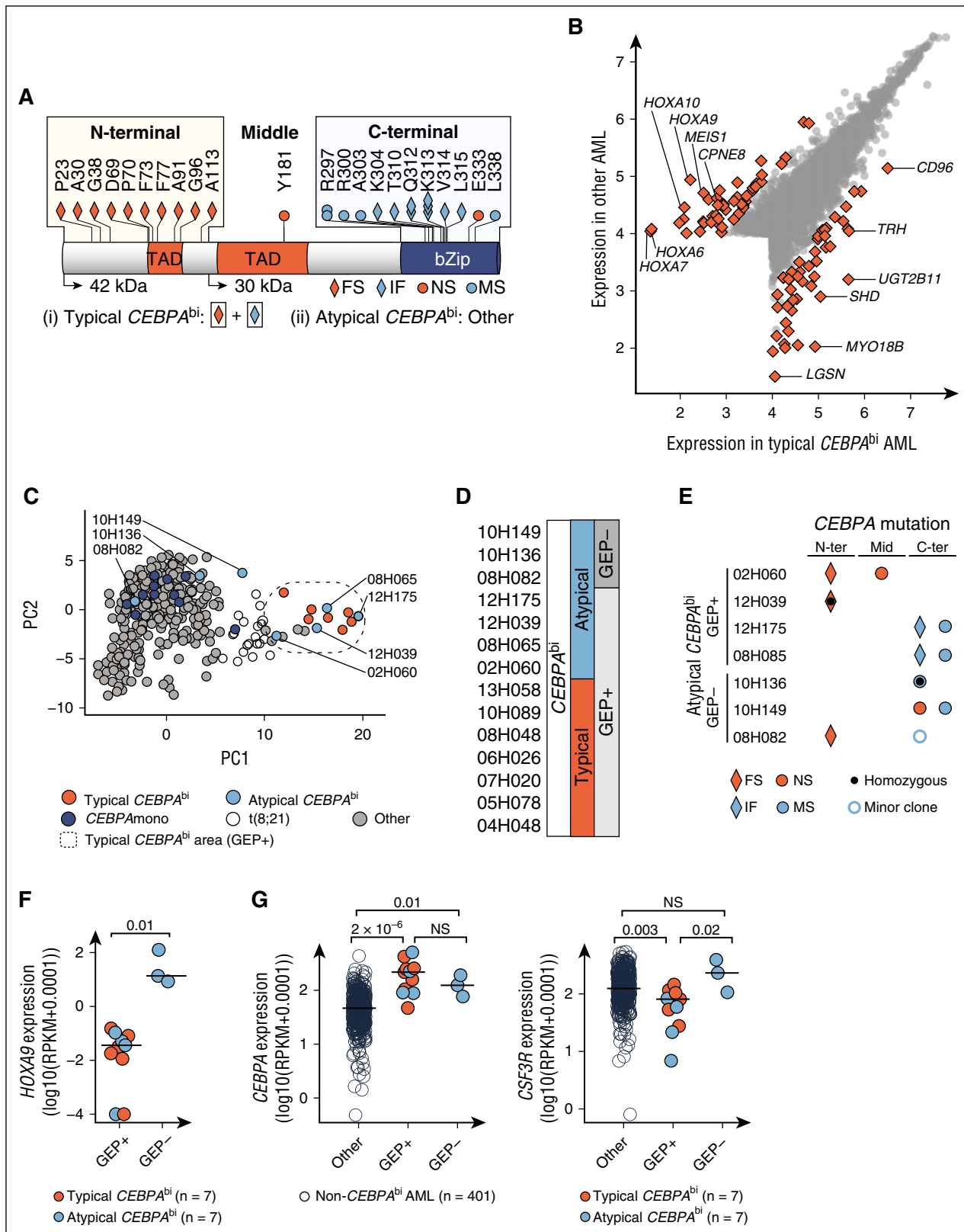
Fourteen AML samples with *CEBPA*<sup>bi</sup> mutations were identified in the Leucegene cohort, including 7 typical and 7 atypical *CEBPA*<sup>bi</sup> AML, comprising 3.4% (14/415) of this collection. Positions of mutations are illustrated in Figure 1A and detailed in supplemental Table 3. Baseline characteristics of cohorts are indicated in Table 1. *CEBPA*<sup>bi</sup> samples were significantly associated with intermediate risk cytogenetics, French-American-British (FAB) M1 morphology, and higher white blood cell counts.

### Typical *CEBPA*<sup>bi</sup> AML shows a uniform GEP

The typical *CEBPA*<sup>bi</sup> specimens are best characterized by a total of 95 genes (Figure 1B; supplemental Table 4). *CEBPA*<sup>bi</sup> AML was marked by low expression of *HOXA* and *HOXB* genes, *MEIS1* and *CPNE8*, a profile partially comparable to that determined for t(8;21) specimens (supplemental Figure 1). Likewise, both leukemia subgroups express high levels of *TRH*, *CD96*, *UGT2B11*, *MYO18B*, and *LSGN*, whereas other genes, such as *SHD*, were most specific to *CEBPA*<sup>bi</sup> AML. This signature is partially consistent but also additive to other signatures published, such as AML cluster 4 reported by Valk et al.<sup>16</sup>

### Gene expression and mutation profile help categorize atypical *CEBPA*<sup>bi</sup> AML

Using the 95-gene signature derived from typical *CEBPA*<sup>bi</sup> AML, we next performed a PCA on the entire Leucegene cohort and confirmed the distinctive GEP of typical *CEBPA*<sup>bi</sup> samples compared with other



**Figure 1. Gene expression signature of *CEBPA*<sup>bi</sup> AML reclassifies atypical *CEBPA*<sup>bi</sup> AML.** (A) Primary structure of *CEBPA* and positions of mutations in *CEBPA*<sup>bi</sup> samples. (B) Comparative analyses of differentially expressed genes in a typical *CEBPA*<sup>bi</sup> AML subgroup. Diamonds correspond to the 95 most differentially expressed genes listed in supplemental Table 4. Genes with low expression in both groups, defined by a mean (log<sub>10</sub> [(RPKM + 0.0001) × 10 000]) <4 (corresponding approximately to 1 RPKM) were not included in this analysis. (C) PCA performed on the entire cohort (n = 415) using the 95-gene signature that characterizes typical *CEBPA*<sup>bi</sup> AML (orange dots). Atypical *CEBPA*<sup>bi</sup> samples (light blue dots) that cluster with typical *CEBPA*<sup>bi</sup> AML (defined by the dashed line) are grouped under GEP+ *CEBPA*<sup>bi</sup> AML, whereas others are termed GEP- *CEBPA*<sup>bi</sup> AML. (D) Classification of *CEBPA*<sup>bi</sup> AML based on *CEBPA* mutations and gene expression. (E) Representation of *CEBPA* mutations in atypical GEP+ or GEP- *CEBPA*<sup>bi</sup> AML. (F) *HOXA9* expression in GEP+ and GEP- *CEBPA*<sup>bi</sup> AML samples. (G) Gene expression of *CEBPA* (left) and *CSF3R* (right) in the entire 415 AML sample cohort. C-ter, C-terminal; *CEBPA*Amono, monoallelic *CEBPA* mutation; IF, inframe; Mid, middle; MS, missense; NS, nonsense; N-ter, N-terminal; PC, principal component.

**Table 1. Characteristics of *CEBPA*<sup>bi</sup> and non-*CEBPA*<sup>bi</sup> AML cohorts**

	<i>CEBPA</i> <sup>bi</sup> (n = 14)	Non- <i>CEBPA</i> <sup>bi</sup> (n = 401)	P
T-AML	0	26 (6.5%)	—
<b>Gender</b>			
Male	9 (64.3%)	226 (56.4%)	—
Female	5 (35.7%)	175 (43.6%)	—
<b>Age (y)</b>			
Median	54.5	58	—
Range	27-84	17-87	—
<b>WBC count (× 10<sup>9</sup>/L)</b>			
Median	88	31	.04
Range	4-177	0.8-361.2	—
<b>Karyotype</b>			
Intermediate	12 (85.7%)	207 (51.6%)	.006
NK	7 (50%)	125 (31.2%)	—
Non-intermediate	2 (14.3%)	192 (47.9%)	—
Undetermined	0	2 (0.5%)	—
<b>FAB subtype</b>			
M0	0	27 (6.7%)	—
M1	8 (57.1%)	107 (26.7%)	.03
M2	3 (21.4%)	49 (12.2%)	—
M3	0	15 (3.7%)	—
M4	0	57 (14.2%)	—
M5	0	66 (16.5%)	—
M6	0	10 (2.5%)	—
M7	0	3 (0.7%)	—
Not classifiable	3 (21.4%)	67 (16.7%)	—
<b>Type of <i>CEBPA</i> mutation</b>			
Biallelic typical	7 (50%)	—	—
Biallelic atypical	7 (50%)	—	—
Monoallelic	—	11 (2.7%)	—

Samples classified under “typical *CEBPA*<sup>bi</sup>” mutations have a combination of heterozygous N-terminal FS or nonsense and C-terminal in frame mutations. Other combinations are considered “atypical *CEBPA*<sup>bi</sup>.”

P values are based on 2-tailed Fisher's exact test or Wilcoxon rank-sum test. T-AML, therapy-related AML; WBC, white blood cell counts.

leukemias, and to specimens with *CEBPA* monoallelic mutations (orange and dark blue dots in Figure 1C). This analysis also showed that 4 out of 7 atypical *CEBPA*<sup>bi</sup> samples clustered with the *CEBPA*<sup>bi</sup> (light blue dots in dashed zone, Figure 1C), suggesting they are transcriptionally similar to typical *CEBPA*<sup>bi</sup> leukemia (hereafter grouped under GEP+ and schematized in Figure 1D). No recurrent *CEBPA* mutation pattern was found in the 4 atypical *CEBPA*<sup>bi</sup> GEP+ specimens, thus suggesting that expression profiling may help to categorize such patients (Figure 1D-E). *HOXA* (eg, *HOXA9*) gene expression may be sufficient to identify *CEBPA*<sup>bi</sup> GEP+ specimens in clinical settings, ie, any *HOXA*<sup>low</sup> atypical *CEBPA* specimen is likely similar to typical *CEBPA*<sup>bi</sup> AML (Figure 1F). The 3 *CEBPA*<sup>bi</sup> GEP– samples were associated with additional genetic rearrangements and/or mutations, which are more typical of non-*CEBPA*-mutated AML (supplemental Table 5). *CEBPA* expression is higher in all *CEBPA*<sup>bi</sup> AML, but more significantly in *CEBPA*<sup>bi</sup> GEP+ AML, in line with the published positive auto-regulatory loop of this transcription factor (left panel in Figure 1G). *CSF3R* expression is lower in *CEBPA*<sup>bi</sup> GEP+ specimens only (right panel in Figure 1G).

### ***CSF3R/STAT5* mutations are the most frequent mutations in *CEBPA*<sup>bi</sup> AML**

We next investigated the mutations in typical and atypical *CEBPA*<sup>bi</sup> cases. A total of 22 additional genes were mutated in this subset: *CSF3R* (4/14, 29%), *WT1*, *GATA2*, and *RUNX1* (3/14), *DNMT3A* and *ASXL1* (2/14), *STAT5B*, *FLT3*, *KRAS*, *NPM1*, *IDH1*, *TET2*, *PTPN11*, *NRAS*, *RAD21*, *SMC1A*, *STAG2*, *U2AF1*, *ZRSR2*, *EZH2*, *CREBBP*, and

*KMT2D* (1/14) (Figure 2A; supplemental Table 6). These results are consistent with other targeted mutation analyses also reporting recurrent *GATA2* and *WT1* mutations in the *CEBPA*<sup>bi</sup> AML subgroup (3/14 vs 10/401, *P* = .007 and 3/14 vs 22/401, *P* = .045, respectively).<sup>19-22</sup> The only *STAT5B* mutation in this cohort was in the *CEBPA*<sup>bi</sup> sample (1/14 vs 0/401) and consisted of an N642H substitution, which is known to increase *STAT5* transcriptional activity and phosphorylation (Figure 2B).<sup>28</sup> *STAT5B* mutations have been described in large granular lymphocytic leukemias<sup>28</sup> and in other T-cell neoplasms,<sup>29,30</sup> but not in AML.

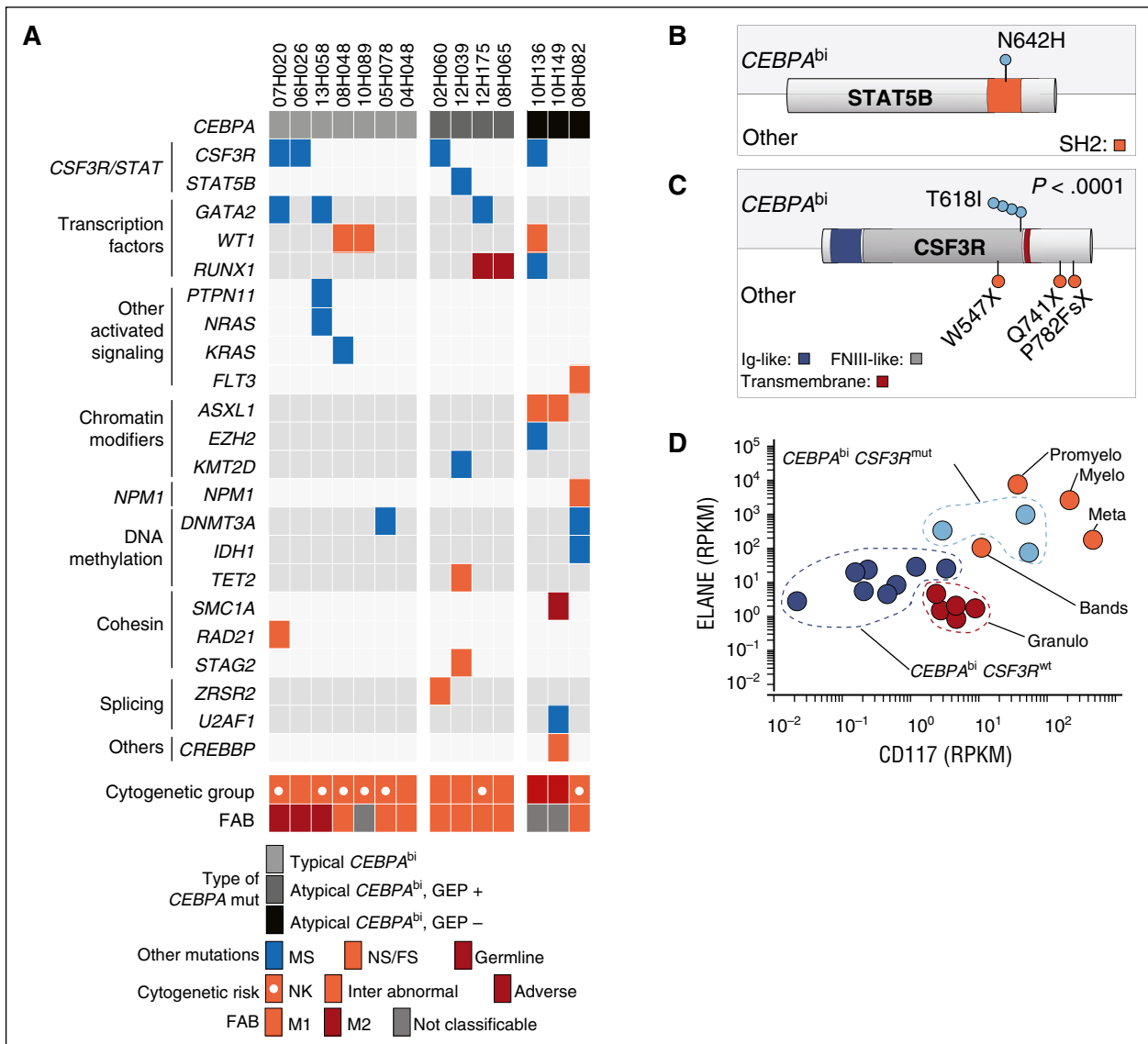
The most frequent mutations in *CEBPA*<sup>bi</sup> subgroup affected *CSF3R* in 4/14 (29%) (Figure 2A; supplemental Figure 2). *CSF3R* mutations were not previously reported in *CEBPA*<sup>bi</sup> AML and they were strongly associated with this subgroup, as only 3 additional *CSF3R* mutations were identified in the entire cohort (4/14 vs 3/401, *P* < .0001; Figure 2C). *CSF3R* T618I “membrane proximal” mutation characterized all 4 *CEBPA*<sup>bi</sup> samples. In contrast, this specific point mutation was not found in the 3 non-*CEBPA* specimens, which carried nonsense or FS *CSF3R* mutations (Figure 2C; supplemental Table 7). A single T618I mutation was observed in The Cancer Genome Atlas in a sample with *CEBPA* monoallelic mutation.<sup>7</sup> *CSF3R* mutations were also found in 1.9% of pediatric AML in an analysis that did not perform *CEBPA* mutational analysis.<sup>8</sup> Rare *CSF3R* T618I mutations were also identified in AML samples with no *CEBPA* mutation, indicating that they can also occur at a low frequency in other genetic contexts.<sup>31</sup> VAF analysis suggests that co-occurrence of *CSF3R* and *CEBPA* mutations were found in the dominant clone (supplemental Table 8).

*CSF3R*<sup>T618I</sup> mutated *CEBPA*<sup>bi</sup> specimens did not show any distinctive clinical laboratory features (supplemental Table 9) but presented a defined transcriptomic profile (supplemental Figure 3) when compared with their WT *CSF3R* counterparts. Gene ontology term enrichment analyses showed a marked enrichment in defense and organisms response genes (supplemental Table 10). In particular, these specimens expressed significantly higher levels of genes associated with myeloid maturation, such as *ELANE* and *CD117*, which upon analysis of larger patient cohorts may become useful in identifying *CSF3R* mutated samples (Figure 2D).

### **Chemical interrogation of *CEBPA*<sup>bi</sup> and *CSF3R* mutated AML**

*CSF3R* signals predominantly through the JAK-STAT pathway.<sup>3</sup> Considering the high frequency of activating T618I *CSF3R* mutations detected in *CEBPA*<sup>bi</sup> AML, we conducted a targeted chemical screen employing a collection of compounds enriched for JAK inhibitors (n = 6; supplemental Table 11). For this study, we used our recently described culture system that preserves the integrity of leukemia blasts and leukemia stem cell activity.<sup>27</sup> Cytotoxic activity of the selected molecules was measured in dose-response studies on a total of 28 primary AML specimens, including all 14 *CEBPA*<sup>bi</sup> AML samples described in the previous sections and 14 randomly selected *CEBPA*<sup>wt</sup> NK controls (supplemental Table 12).

Responses of *CEBPA*<sup>bi</sup> specimens (n = 14) and NK *CEBPA*<sup>wt</sup> AML (n = 14) to the positive controls cytarabine and daunorubicin were comparable (Figure 3A-B and supplemental Figure 4A, compounds IC<sub>50</sub> in supplemental Table 13, and *P* values in supplemental Table 14). Likewise, these 2 AML subgroups were equally sensitive to the multikinase inhibitors sorafenib and dasatinib reported to lack JAK inhibitory activity (Figure 3A,C; supplemental Figure 4B). In contrast, *CEBPA*<sup>bi</sup> AML were statistically more sensitive than control NK AML to inhibitors which more specifically targeted JAK proteins (ruxolitinib, CYT387, tofacitinib, and AZD1480) (Figure 3A,D; supplemental Figure 4C). Although of high potency, compounds such as lestaurtinib



**Figure 2. Mutational landscape of *CEBPA*<sup>bi</sup> AML.** (A) Mutation profile of *CEBPA*<sup>bi</sup> AML. Samples are grouped together according to the type of *CEBPA* mutations: typical *CEBPA*<sup>bi</sup>, atypical GEP+ *CEBPA*<sup>bi</sup>, and atypical GEP− *CEBPA*<sup>bi</sup>, illustrated by shades of gray from light to dark. Each column represents a patient sample. (B-C) Primary structures of STAT5B (B) and CSF3R (C) proteins with corresponding positions of mutations in *CEBPA*<sup>bi</sup> and non-*CEBPA*<sup>bi</sup> (other) AML. (D) Expression of *ELANE* and *CD117* in GEP+ *CEBPA*<sup>bi</sup> compared with normal sorted granulocyte populations from peripheral blood (n = 5, red) and normal marrow precursors (n = 4, orange). FNIII, fibronectin type III; Granulo, granulocytes; Ig, immunoglobulin; Inter, intermediate; Meta, metamyelocytes; Mut, mutated; Myelo, myelocytes; NPM1, nucleophosmin 1; Promyelo, promyelocytes.

and TG-101348 that target JAK and other kinases were less discriminatory (red dots in Figure 3A and supplemental Figure 4D).

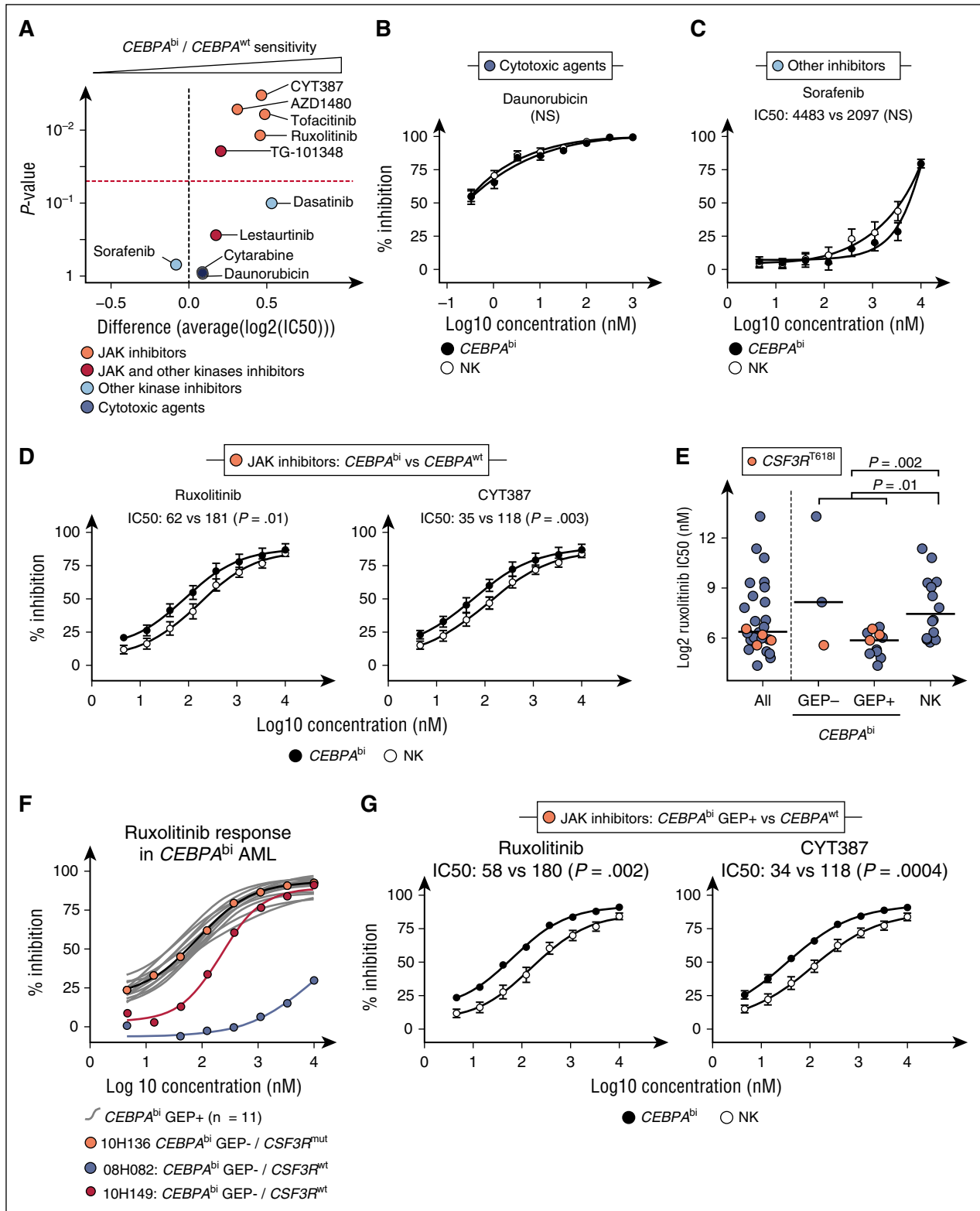
Specimens with activating mutations in *CSF3R* were among the most sensitive to ruxolitinib (orange dots in Figure 3E) and to the other 3 most specific JAK inhibitors (supplemental Figure 5). Of the 3 *CEBPA*<sup>bi</sup> GEP− samples, the highest sensitivity to JAK inhibitors was observed for the *CSF3R*-mutated specimen (Figure 3E, second column and Figure 3F; see also supplemental Figure 6 for other JAK inhibitors). These results are in agreement with those of Maxson et al,<sup>3</sup> who reported a correlation between sensitivity to JAK inhibitors and *CSF3R*<sup>T618I</sup> mutation in 2 other related leukemias, T acute lymphoblastic leukemia and chronic neutrophilic leukemia.

#### ***CEBPA*<sup>bi</sup> GEP+ but not GEP− AML are homogeneously sensitive to JAK inhibitors**

The 11 *CEBPA*<sup>bi</sup> GEP+ specimens tested, irrespective of their *CSF3R* mutation status, were equally sensitive to the JAK inhibitors (Figure 3E,

third column for ruxolitinib). The uniform response to JAK inhibition in the homogeneous *CEBPA*<sup>bi</sup> GEP+ subgroup stands in sharp contrast to the heterogeneous response determined for the NK control group (Figure 3E, fourth column) and the GEP− specimens, and strengthens our hypothesis that the *CEBPA*<sup>bi</sup> GEP− and GEP+ are distinct entities (Figure 3G; supplemental Figure 7).

These results suggest that networks or pathways upstream of JAK-STAT are aberrantly activated in the majority of *CEBPA*<sup>bi</sup> GEP+ specimens and, less frequently in other NK AML. Activating mutations in *CSF3R* could account for responses in 3 out of 11 *CEBPA*<sup>bi</sup> GEP+, but not for either the remaining 8 *CEBPA*<sup>bi</sup> GEP+ specimens and/or the sensitive NK AML specimens (supplemental Table 15). To further investigate this observation, we systematically analyzed all variants present in genes (n = 167) relevant to the JAK network, excluding its proposed downstream targets (supplemental Table 2). In the addition of the *CSF3R* T618I mutation described above, we detected 2 recurrent variants, *JAK2* L383V and *EPHB6* S166F, characterized by a 7.4- and



**Figure 3. *CEBPA*<sup>bi</sup> AML is uniformly sensitive to JAK inhibitors.** (A) Volcano plot showing the comparative sensitivity of *CEBPA*<sup>bi</sup> AML (n = 14) vs *CEBPA*<sup>wt</sup> NK AML (n = 14) to selected small molecules. Dashed red line corresponds to *P* = .05. (B–D) Mean dose-response curves with SEM and IC<sub>50</sub>-associated statistics comparing *CEBPA*<sup>bi</sup> AML (n = 14) to *CEBPA*<sup>wt</sup> NK AML (n = 14) for selected compounds as follows: (B) daunorubicin, (C) sorafenib, and (D) ruxolitinib and CYT387. Results for other compounds are shown in supplemental Figure 4. (E) IC<sub>50</sub> for ruxolitinib in all samples (n = 28) (left), *CEBPA*<sup>bi</sup> GEP– AML (n = 3) and *CEBPA*<sup>bi</sup> GEP+ AML (n = 11) (middle), and NK AML (n = 14) (right). *CSF3R*<sup>T618I</sup> mutated samples are indicated in orange. Horizontal bars represent medians. (F) Individual dose-response curves (gray) for GEP+ and GEP– (*CEBPA*<sup>bi</sup> AML). Results for other JAK inhibitors are shown in supplemental Figure 6. (G) Mean dose-response curves with SEM and IC<sub>50</sub>-associated statistics comparing *CEBPA*<sup>bi</sup> GEP+ AML (n = 11) to *CEBPA*<sup>wt</sup> NK AML (n = 14) for selected JAK inhibitors. Results for other JAK inhibitors are shown in supplemental Figure 7. *P* values were calculated using a Wilcoxon rank-sum test on IC<sub>50</sub> values. NS, not significant; SEM, standard error of the mean.

>117-fold observed/expected enrichment ratios, respectively (supplemental Table 16). Both variants were confirmed to be germ line. Interestingly, the S166F substitution within the ligand-binding domain of EPHB6 is predicted to affect the ligand binding and consequently activity of this receptor. Moreover, this variant allele detected exclusively in 2 *CEBPA*<sup>bi</sup> GEP+ samples has never been described before. We also found a large number of nonrecurrent variants in several cytokine receptors in these specimens (supplemental Table 16), but larger patient cohorts would be required to select potential candidates for functional validation studies.

In conclusion, this study documents for the first time recurrent activating *CSF3R* mutations in AML with a strict association to the rare *CEBPA*<sup>bi</sup> genetic subgroup. Targeted analyses in other cohorts will precise the frequency of *CSF3R* mutation in *CEBPA*<sup>bi</sup> and non-*CEBPA*<sup>bi</sup> AML as well. Of interest, Maxson et al recently presented similar findings.<sup>32</sup> As might be anticipated, these *CSF3R* mutated specimens are sensitive to JAK inhibition. Most notably, our study also shows that *CEBPA*<sup>bi</sup> GEP+ AMLs are uniformly sensitive to JAK inhibition, raising the possibility that selective genetic pressure resulted in a dependence on the JAK-STAT signaling pathway. An unexpected frequency of variant alleles in JAK-STAT network genes provides a pipeline for future exploration. Considering that most molecules tested herein are available drugs, these studies suggest that JAK inhibitor repositioning could thus represent a true example of therapy targeting a specific well-defined subset of AML patients.

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## Authorship

Contribution: V.-P.L. contributed to project conception, analyzed NGS and chemical screen data, generated all figures, tables, and supplementary material, and was the main author of this paper; G.S. contributed to project conception and coordination and cowrote the paper; J.H. contributed to project conception, analyzed the cytogenetic and fluorescence in situ hybridization studies, provided all the AML samples, and edited the manuscript; P.G. processed the raw NGS data; G.B. codeveloped the analytical pipeline; S.L. was responsible for supervision of the bioinformatics team and of statistical analyses; I.B. performed data validation and chemical screen; J.K. conceived and performed the chemical screen; A.M. was responsible for the chemistry team as part of the Leucegene project and analyzed the chemical screen data; S.M. contributed to the selection of compounds and interpretation of results with C.J.G.; and C.P. processed and sequenced normal peripheral and BM populations.

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