

Tribbles homolog 2 inactivates C/EBP α and causes acute myelogenous leukemia

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Summary

Tribbles homolog 2 (Trib2) was identified as a downregulated transcript in leukemic cells undergoing growth arrest. To investigate the effects of Trib2 in hematopoietic progenitors, mice were reconstituted with hematopoietic stem cells retrovirally expressing Trib2. Trib2-transduced bone marrow cells exhibited a growth advantage ex vivo and readily established factor-dependent cell lines. In vivo, Trib2-reconstituted mice uniformly developed fatal transplantable acute myelogenous leukemia (AML). In mechanistic studies, we found that Trib2 associated with and inhibited C/EBP α . Furthermore, Trib2 expression was elevated in a subset of human AML patient samples. Together, our data identify Trib2 as an oncogene that induces AML through a mechanism involving inactivation of C/EBP α .

Introduction

Acute myelogenous leukemia (AML) is a genetically and phenotypically heterogeneous disease that is characterized by a block in myeloid differentiation, as well as enhanced proliferation and survival (Kelly and Gilliland, 2002). AML is frequently associated with chromosomal translocations that target transcription factors such as members of the core binding factor (CBF) family, resulting in fusion proteins that include AML1/ETO [t(8;21)], CBF β /SMMHC [inv(16)], and TEL/AML1 [t(12;21)]. Mutations in transcription factors such as *PU.1*, *C/EBP α* , *AML1*, and *GATA-1* are also associated with AML, as are other types of oncogenic perturbations that lead to functional inactivation of critical transcription factors (Mueller and Pabst, 2006; Rosenbauer et al., 2005; Tenen, 2003). Recurrent involvement of a limited set of transcription factors suggests that disruption of these genes is rate limiting for leukemia development.

Tribbles was first identified in *Drosophila* as a gene that is required for gastrulation, oogenesis, and viability (Grosshans and Wieschaus, 2000; Mata et al., 2000; Seher and Leptin, 2000).

Tribbles coordinates cell division during gastrulation by promoting turnover of the cell cycle protein String/CDC25, thereby inhibiting premature mitosis. In this context, loss of *Tribbles* function was associated with increased proliferation, whereas overexpression of *Tribbles* slowed the cell cycle (Mata et al., 2000). *Tribbles* also promotes the degradation of *slbo*, the *Drosophila* homolog of the C/EBP family of basic region-leucine zipper transcription factors, during oogenesis (Rorth et al., 2000). Based on amino acid sequence, *Tribbles* resembles a serine-threonine kinase; however, it has a variant catalytic core that lacks a canonical ATP binding site, and how it functions is unknown.

Mammals have three homologs of *Tribbles*—*Trib1*, *Trib2*, and *Trib3*. During fasting conditions, *Trib3* negatively regulates AKT in the liver (Du et al., 2003) and binds the E3 ligase COP1 to degrade ACC in adipose tissue (Qi et al., 2006). It is also a transcriptional target of the nuclear hormone receptor PPAR α (Koo et al., 2004). These findings are consistent with a function downstream of the insulin receptor. *Trib3* is also upregulated in tumor cells and in hypoxic conditions (Bowers et al., 2003). Less is known

SIGNIFICANCE

AML, a heterogeneous disease characterized by increased proliferation and a block in differentiation, is commonly associated with chromosomal translocations and transcription factor perturbations. We identified Trib2 as an oncogene that efficiently produces AML in mice. Retroviral Trib2 expression in hematopoietic stem cells perturbed myeloid development, enhanced progenitor proliferation, and directly inhibited the function of C/EBP α , a critical transcription factor that is frequently dysregulated in AML. Trib2 expression was upregulated in a subset of primary patient-derived AML samples, suggesting that it also contributes to the development of human AML. The identification of Trib2 as a potent leukemogen points to alternative pathogenic mechanisms and possible therapeutic opportunities in this aggressive cancer, which is not currently curable in the majority of patients.

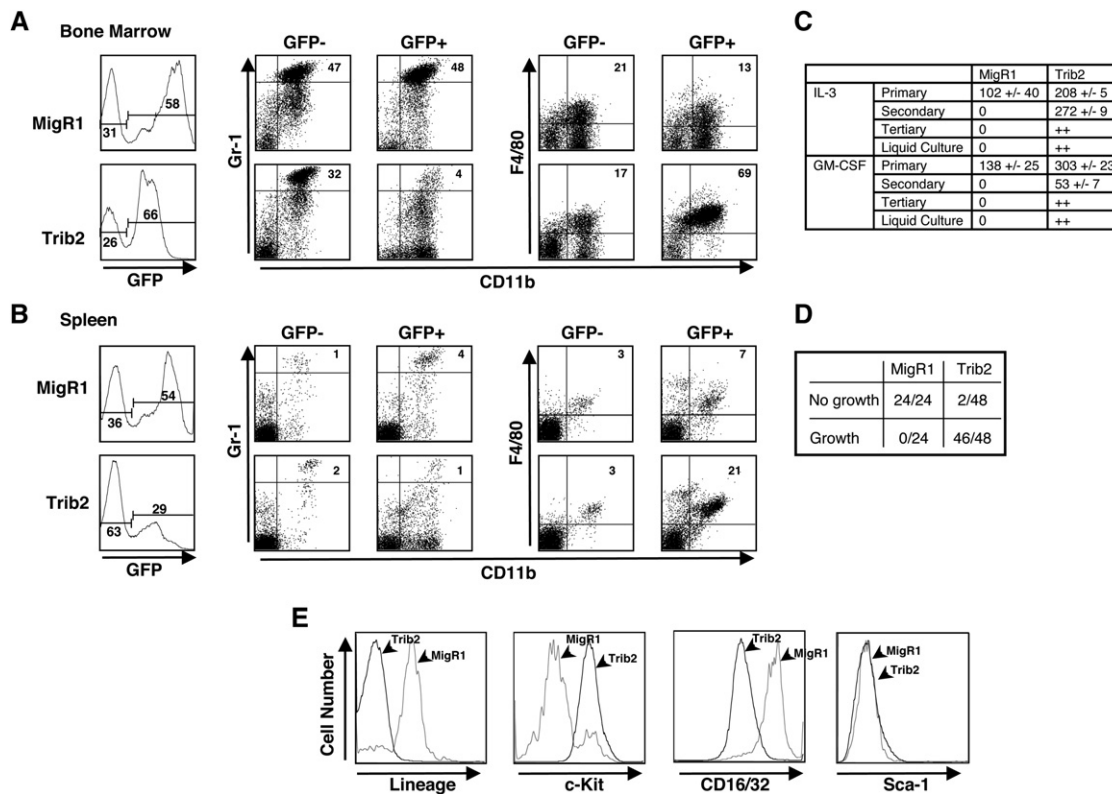


Figure 1. Trib2 perturbs myeloid development in vivo and promotes self-renewal in vitro

B6 mice were lethally irradiated and reconstituted with BM cells transduced with control MigR1 vector or Trib2. Flow cytometric analysis of MigR1 and Trib2 chimeric mice at 9–14 weeks post-BMT.

A and B: BM (**A**) and spleen (**B**) showing GFP positivity and Gr-1^{hi}CD11b⁺ neutrophils (percentages given) in the GFP⁺ and GFP⁻ fractions (left panel). In the right panel, F4/80⁺CD11b⁺ monocytes (percentages given) in the GFP⁺ and GFP⁻ fractions are shown. Results are representative of three independent experiments using six mice.

C: GFP⁺ BM cells (25,000) sorted from MigR1 and Trib2 chimeric mice at week 9 post-BMT were plated in methylcellulose in the presence of the indicated cytokines. Colonies with >50 cells were scored after primary plating (9 days), secondary replating (15,000 cells, 10 days), tertiary replating (15,000 cells, 8 days), and growth in liquid culture (8 days) in triplicate. The mean numbers of colonies, \pm SEM, for each condition are tabulated. “++” indicates colony growth or growth in liquid culture.

D: Five thousand sorted GFP⁺, lin⁻ BM cells from Trib2 and MigR1 chimeras were plated in triplicate in methylcellulose containing IL-3, IL-6, SCF, and GM-CSF. Single colonies from primary MigR1 and Trib2 plates at day 9 were replated in liquid culture plus cytokines (IL-3, IL-6, SCF) in 24-well plates and assessed for growth after 11 days (growth = cell expansion and yellow media).

E: FACS analysis of MigR1 and Trib2 cells from a representative liquid culture in **D**.

about the other two mammalian Trib family members. When overexpressed, Trib1 can inhibit MEKK-1-mediated activation of AP-1 and stress kinase signaling (Hegedus et al., 2006; Kiss-Toth et al., 2004). Molecular data for Trib2, originally identified as a phosphoprotein in canine thyroid cells (Wilkin et al., 1997), are limited to a study identifying it as a possible autoantigen in autoimmune uveitis (Zhang et al., 2005) and as a differentially expressed gene in kidney mesenchymal cells and in a prostate cancer cell line (Bisoffi et al., 2004; Takasato et al., 2004).

To investigate Trib2's function in hematopoiesis, we retrovirally expressed it in murine hematopoietic progenitors, which were studied for changes in growth and differentiation in vivo and in vitro. This led to long-term growth of myeloid progenitors in vitro and induction of AML in vivo. Our biochemical studies suggest that an important function of Trib2 in transformation is to inactivate C/EBP α . Furthermore, an analysis of microarray data generated from 285 AML patient samples identified elevated *Trib2* expression in a distinct subset of patients in a cluster with a high frequency of C/EBP α mutations. Together, these

data identify Trib2 as a leukemogenic oncogene with the capacity to dysregulate C/EBP α signaling.

Results

Trib2 perturbs myeloid development in vivo

We identified *Trib2* as a transcript that was downregulated after treatment of Notch-dependent murine T-ALL cell lines with γ secretase inhibitors (unpublished data). As little was known about Trib2 function, we initiated studies to determine its effects on hematopoiesis. Lethally irradiated C57BL/6 (B6) mice were reconstituted with Trib2 or retroviral control vector (MigR1)-transduced hematopoietic progenitors as described (Pui et al., 1999). At 9–14 weeks post-bone marrow transplant (BMT), MigR1 and Trib2 chimeric mice were similar in terms of bone marrow (BM) and splenic cellularity, the overall percentage of GFP⁺ cells, and the peripheral white blood cell (WBC) counts (Figures 1A and 1B, left panels). However, granulocytes (CD11b⁺Gr-1^{hi}) were reduced in the GFP⁺ BM population of

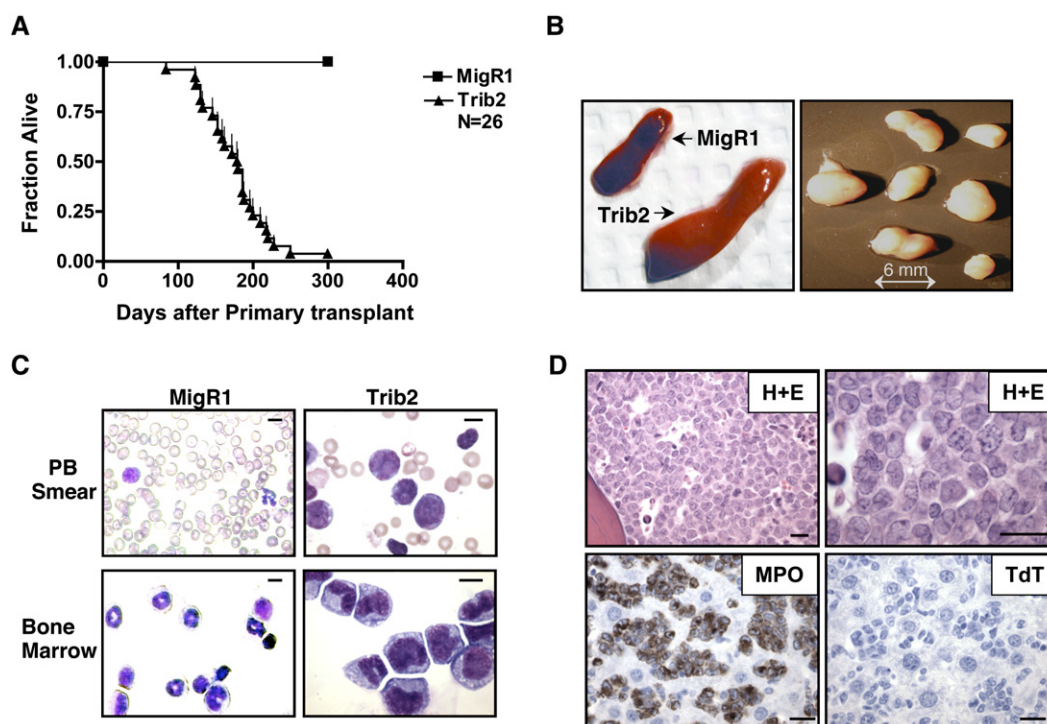


Figure 2. Trib2 induces AML

A: Kaplan-Meier survival curve of mice receiving Trib2-transduced BM compared to MigR1 control. The median survival of Trib2 mice was 179 days. Results are derived from seven independent experiments.

B: Representative photographs of splenomegaly in Trib2 mice compared to control MigR1 spleen, and lymphadenopathy in Trib2 mice.

C: Wright-Giemsa-stained PB and BM single cell suspensions from MigR1 and leukemic Trib2 mice. Scale bars (upper right) represent 10 μ m. The percentage of GFP⁺ cells in Trib2 BM was approximately 90%–100%.

D: Histopathology of BM sections from Trib2-induced AML. Hematoxylin and eosin (H+E) section showing hypercellularity (top left) due to the presence of sheets of immature cells and blasts (top right). The tumor cells stain positively for myeloperoxidase (MPO, bottom left) and negatively for terminal deoxynucleotidyl transferase (TdT, bottom right). Scale bars (lower right) represent 20 μ m.

Trib2 chimeric mice (Figures 1A and 1B, center panels), and monocytes (CD11b⁺F4/80⁺) were significantly increased in the GFP⁺ population of both the BM and spleen of Trib2 mice (Figures 1A and 1B, right panels), suggesting that enforced expression of Trib2 promotes monocytic differentiation and inhibits granulocytic differentiation. Further characterization of myeloid subsets showed the presence of increased numbers of CD11b⁺CD11c⁺MHCII⁺ dendritic cells (DCs) and CD11b⁺F4/80⁺MHCII⁺ macrophages in the GFP⁺ population of Trib2 chimeric mice as compared to MigR1 control mice (Figures S1A and S1B in the Supplemental Data available with this article online). In contrast to myeloid development, Trib2-GFP⁺ lymphoid cells obtained from the BM, lymph nodes, and thymus did not reveal developmental abnormalities (data not shown). These experiments suggest that enforced Trib2 expression perturbed myeloid development.

Trib2-transduced progenitors exhibit a growth advantage in vitro and establish long-term myeloid progenitor cell lines

To assay the proliferative potential of Trib2-transduced cells in vitro, sorted GFP⁺ cells from the BM of MigR1 and Trib2 chimeras at 9–10 weeks post-BMT were plated in methylcellulose in the presence of IL-3 or GM-CSF. Significant differences in colony numbers were observed in both IL-3 and GM-CSF (Figure 1C). When replated, Trib2 cells formed secondary colonies

in both IL-3 and GM-CSF, whereas MigR1 primary colonies did not. Cells from Trib2 secondary colonies formed tertiary colonies, and cells transferred from these colonies to liquid culture exhibited factor-dependent (IL-3 and GM-CSF) long-term growth (Figure 1C). Lineage-depleted GFP⁺ BM progenitor cells sorted from MigR1 and Trib2 chimeras were then assayed for proliferation. Single colonies were randomly picked from primary methylcellulose plates and replated in liquid media containing IL-3, IL-6, and SCF. Cells from MigR1 colonies did not proliferate in liquid medium, whereas cells from 46/48 Trib2 colonies exhibited factor-dependent long-term growth in liquid cultures (Figure 1D). These Trib2-expressing cell lines remained factor dependent and did not produce leukemia when injected into recipient mice (data not shown). The proliferating Trib2 cells did not express lineage markers (Gr-1, Ter119, CD3, CD8, CD4, B220, CD19, CD11c, CD11b) or Sca-1 but did express c-Kit and CD16/32 (Fc γ III/II) as compared to MigR1 control cells (Figure 1E). These data suggest that Trib2 expression in myeloid progenitors promotes replating and self-renewal activities.

Mice reconstituted with Trib2 bone marrow cells develop AML

Trib2 chimeric mice died prematurely at a median of 179 days post-BMT (Figure 2A and Table S1, one outlier mouse of $n = 26$). These data include mice from multiple independent BMT

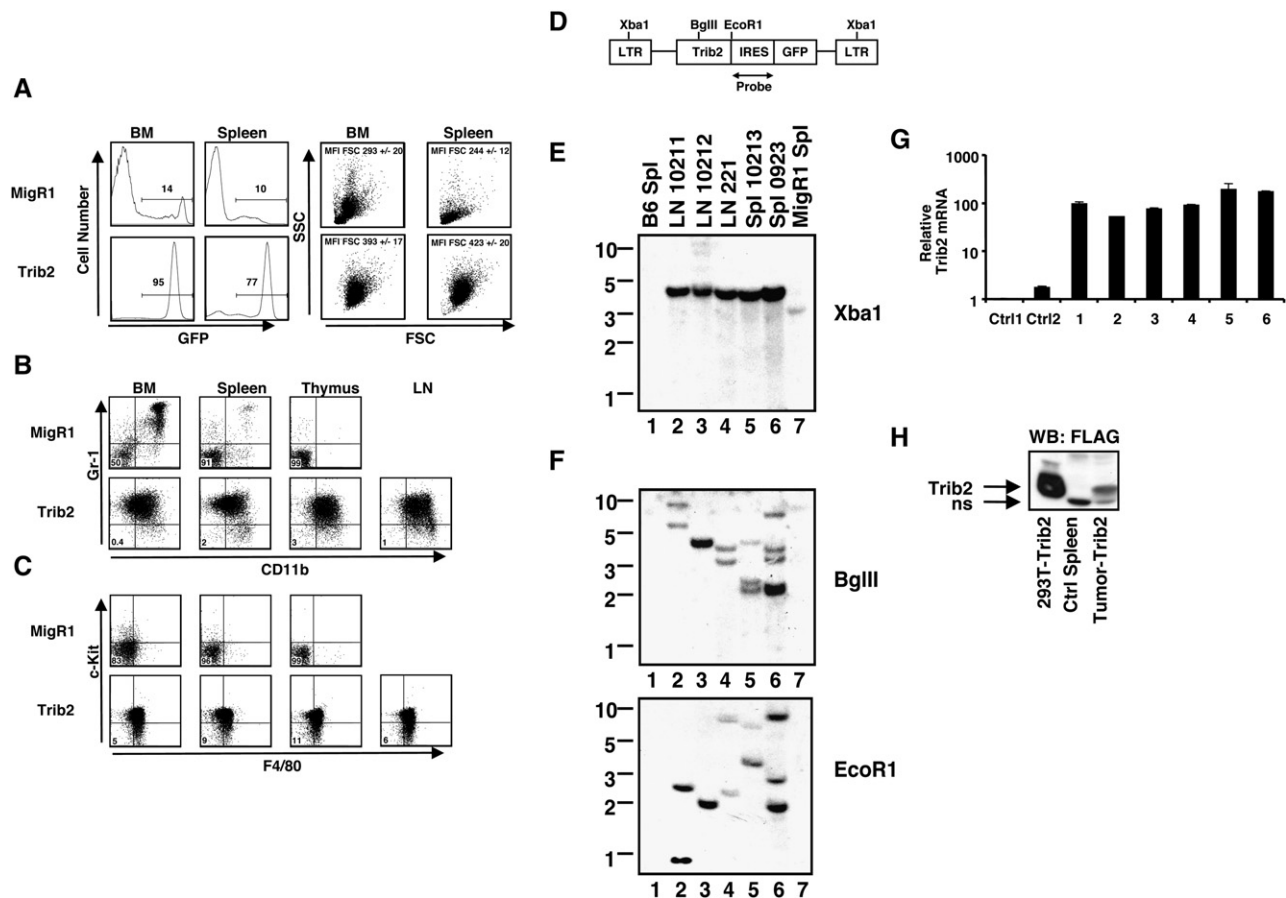


Figure 3. Analysis of Trib2-induced AML

A: Flow cytometric analysis of GFP expression (percentages given), forward scatter (FSC), and side scatter (SSC) in cells obtained from BM and spleens of leukemic mice. Mean fluorescence intensities (MFI) of FSC of the GFP⁺ cells are shown \pm SD.

B and C: Immunophenotypes of primary Trib2-induced AML cells from lymph nodes (LN), thymus, spleen, and BM compared to control MigR1 cells. Flow cytometric analysis of Gr-1 and CD11b expression (**B**) and c-Kit and F4/80 expression (**C**), in the GFP⁺ fraction of MigR1 (top) and Trib2 (bottom panels) mice. The percentages of cells that are Gr-1⁺CD11b⁺ are shown in **B**, and the percentages of cells that are c-Kit⁺F4/80⁺ are shown in **C**. Results are representative of seven independent experiments.

D: Schematic of Trib2 provirus and probe used for Southern blotting.

E and F: Southern blot detection of proviruses in Trib2 mice. DNA preparations were digested with either Xba1 to detect intact provirus (\sim 4 kb) (**E**) or BglII or EcoRI, which cleaved once in the provirus, to detect different proviral integrants (**F**). All leukemic samples were obtained from different primary mice; lanes 1 and 7 contain control DNAs from B6 mouse spleen (Spl) and MigR1 control spleen, respectively.

G: Real-time RT-PCR analysis of Trib2 expression in cDNAs from six primary Trib2-induced AML tumor spleen samples (1–6), using murine Trib2-specific primers. The results are presented as Trib2 mRNA in AML samples relative to expression in control MigR1 spleen cells (Ctrl1, Ctrl2), normalized for 18S rRNA content. Error bars denote \pm SD of each sample measured in triplicate.

H: Analysis of Trib2 protein expression by western blot. Extracts of primary Trib2-induced AML cells obtained from spleen (>90% GFP, MigR1-Trib2-FLAG) were compared to extracts prepared from control MigR1 spleen cells and 293T cells transfected with pcDNA-Trib2-FLAG. Trib2-FLAG polypeptides were detected on the blot with the FLAG antibody (M2, Sigma). ns, nonspecific band.

experiments performed with different Trib2 retroviral constructs (MigR1-Trib2, MigR1-Trib2-Flag-tagged, tNGFR-Trib2-Flag-tagged). The peripheral blood (PB) prior to death exhibited a leukocytosis consisting of blasts and immature myelomonocytic cells (Figure 2C and Table S1). At necropsy, all mice displayed splenomegaly and lymphadenopathy (Figure 2B and Table S1). Morphologically, the infiltrating cells in these tissues resembled those seen in the PB; mature granulocytes were notably absent (Figure 2C). Histologic sections revealed extensive (>80% in all cases) involvement of the BM by myeloblasts and immature myelomonocytic cells (Figure 2D, top panels). Blasts made up >20% of the BM cellularity in all affected animals, supporting the diagnosis of AML. Leukemic cells found in the spleen, liver, lymph nodes, and blood had similar morphologic

and immunophenotypic characteristics (data not shown). By immunohistochemical staining, these cells were positive for myeloperoxidase (a marker of myeloblasts and more mature granulocytes) and were negative for terminal deoxynucleotidyl transferase (a marker of lymphoblasts) (Figure 2D, lower panels). Cytochemical stains for nonspecific esterase, a marker of monocytes, were negative (data not shown).

Flow cytometric analysis revealed that the leukemic cells were GFP⁺ and relatively large in size (as judged by forward scatter, Figure 3A) and expressed intermediate levels of Gr-1 and CD11b (Figure 3B), as well as c-Kit and F4/80 (Figure 3C). The coexpression of Gr-1 and CD11b at low/intermediate levels is characteristic of immature myeloid precursors and murine myeloid leukemias (Cozzio et al., 2003; de Guzman et al., 2002).

The overall combination of morphologic and immunophenotypic findings is consistent with AML.

To detect provirus, DNA was isolated from the lymph nodes and spleens of chimeric mice and analyzed by digestion with restriction endonucleases (Figure 3D). These studies revealed that all tumors contained intact provirus (Figure 3E) and that individual tumors contained one to several distinct proviral insertions (Figure 3F). The intensity of the bands indicated that the tumors were either monoclonal (Figure 3F, lanes 2, 3, and 4) or biclonal (Figure 3F, lanes 5 and 6), suggesting that additional genetic events are necessary to generate Trib2-induced AML. In addition to GFP (Figure 3A), the tumor cells expressed both Trib2 mRNA (Figure 3G) and protein (38 kDa, Figure 3H). Because antibodies against Trib2 are not available, the latter was detected in a cohort of tumors induced with epitope-tagged Trib2 retrovirus.

To further establish the malignancy of Trib2 leukemia, cells (2×10^6) from the BM and spleens of primary leukemic mice were transplanted into sublethally irradiated secondary recipients. All secondary recipients developed AML with an average latency of 36 days (Figure 4A and Table S2). Secondary leukemias were associated with leukocytosis and extensive involvement of the BM, liver, spleen, and other tissues (Figure 4B and Table S2). The immunophenotype of the leukemic cells in secondary recipients resembled that of the primary disease, with most cells retaining intermediate expression of Gr-1 and CD11b (Figure 4B) and expression of F4/80 (Figure 4C). In addition, the leukemic cells were c-Kit⁺, Sca-1⁻, CD34^{intermediate}, and CD16/32⁺ (Fc γ III/II) (Figure 4D). Together, these data indicate that Trib2 is a potent leukemogen.

Trib2 reduces normal C/EBP α levels and inhibits C/EBP α function

A deficiency of the transcription factor C/EBP α inhibits granulocyte differentiation and is associated with AML (Tenen, 2003), features similar to the consequences of Trib2 expression in hematopoietic progenitors. Furthermore, *Drosophila* Tribbles can promote the degradation of Slbo, the *Drosophila* homolog of C/EBP, during oocyte border cell migration (Rorth et al., 2000). To determine whether Trib2 alters C/EBP α protein levels in myeloid cells, western blot analysis was performed on whole-cell extracts prepared from 32D and U937 cells transduced with MigR1 or Trib2. In both cell lines, Trib2 expression reduced levels of the C/EBP α 42 full-length protein and concomitantly increased levels of C/EBP α p30 (Figure 5A), a dominant-negative variant of C/EBP α 42 that is thought to arise from an internal translation initiation site (Calkhoven et al., 2000).

Human AML is associated with mutations in C/EBP α that lead to decreased levels of normal C/EBP α p42 and increased levels of C/EBP α p30 (Leroy et al., 2005; Pabst et al., 2001b). C/EBP α p30 dominantly inhibits C/EBP α p42 function when the ratio of C/EBP α p42 to C/EBP α p30 is less than one (Calkhoven et al., 2000). In contrast to normal CMPs and GMPs (Akashi et al., 2000), high levels of C/EBP α p30 and low ratios of C/EBP α p42 to C/EBP α p30 were present in all Trib2 primary leukemic samples (Figure 5B, left panel). The inverted ratio of C/EBP α p42 to C/EBP α p30 protein persisted in serially passaged leukemic cells (Figure 5B, right panel). The identity of C/EBP α p30 was confirmed by performing additional western blots with an antibody specific for the N-terminal C/EBP α epitope, which detected only C/EBP α p42 (data not shown).

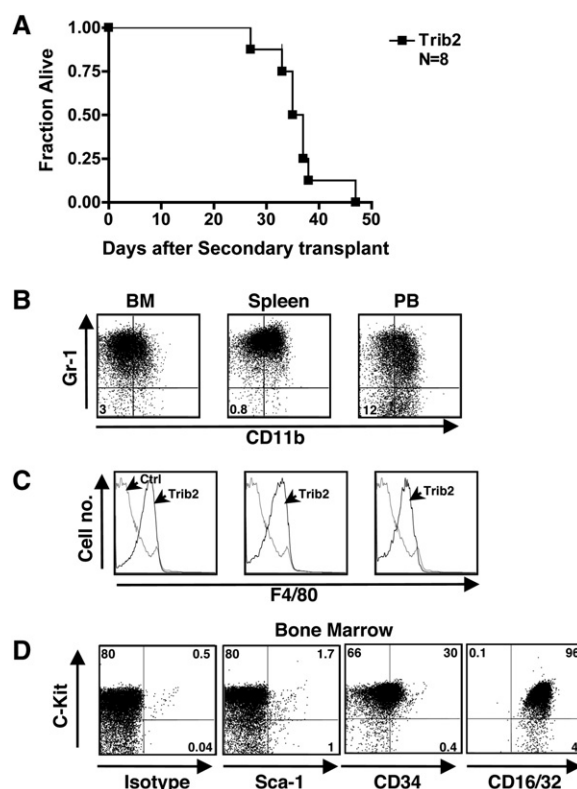


Figure 4. Trib2-induced AML is 100% transplantable

A: Kaplan-Meier survival curve of secondary transplants. Sublethally irradiated mice (600 rads) received 2×10^6 BM or spleen cells from eight primary leukemic mice.

B–D: Immunophenotype of Trib2-induced AML after secondary transplant. Cells from BM, spleen, and PB were assessed by flow cytometry. **B:** The Gr-1 and CD11b profile of the GFP⁺ population is shown; percentages given are cells negative for both markers. **C:** F4/80 profile of the GFP⁺ Trib2 cells (black line) are overlaid on the normal F4/80 staining profile of B6 control BM cells (gray line). Results shown are representative of $n = 8$ mice. **D:** The c-Kit and isotype/Sca-1/CD34/CD16/32 profile of the GFP⁺ population is shown.

To show that the relative decrease in C/EBP α p42 translated into diminished functional activity, we investigated whether Trib2 inhibited activation of an IL-12 promoter luciferase reporter by C/EBP α . This reporter contains a single C/EBP α binding site, which is involved in the induction of IL-12 transcription in RAW cells following LPS exposure (Figure 5C). Trib2 inhibited the stimulation of an IL-12 reporter construct by both C/EBP α p42 and LPS (Figure 5D). In both experiments, the stimulation of reporter gene activity by C/EBP α or LPS required an intact C/EBP α binding site (Figure 5D). Trib2 had no effect on an NF κ B-sensitive luciferase reporter gene (Figure S2A), suggesting that the effect of Trib2 was specific for C/EBP α . Furthermore, the IL-6 promoter also contains a functional C/EBP α binding site, and BM-DCs and macrophages derived from Trib2 chimeric mice released less IL-12 and IL-6 in response to LPS than did MigR1 control cells (Figures S2B–S2E). To further characterize the relationship between C/EBP α and Trib2, the C/EBP α DNA binding activity was assayed in extracts prepared from transduced U937 cells. The C/EBP α -DNA binding activity was markedly reduced by Trib2 (Figure 5E, lanes 1 and 3), with the specificity of the observed gel shift being confirmed by production of a “supershift” upon addition of an antibody against C/EBP α (Figure 5E, lane 2

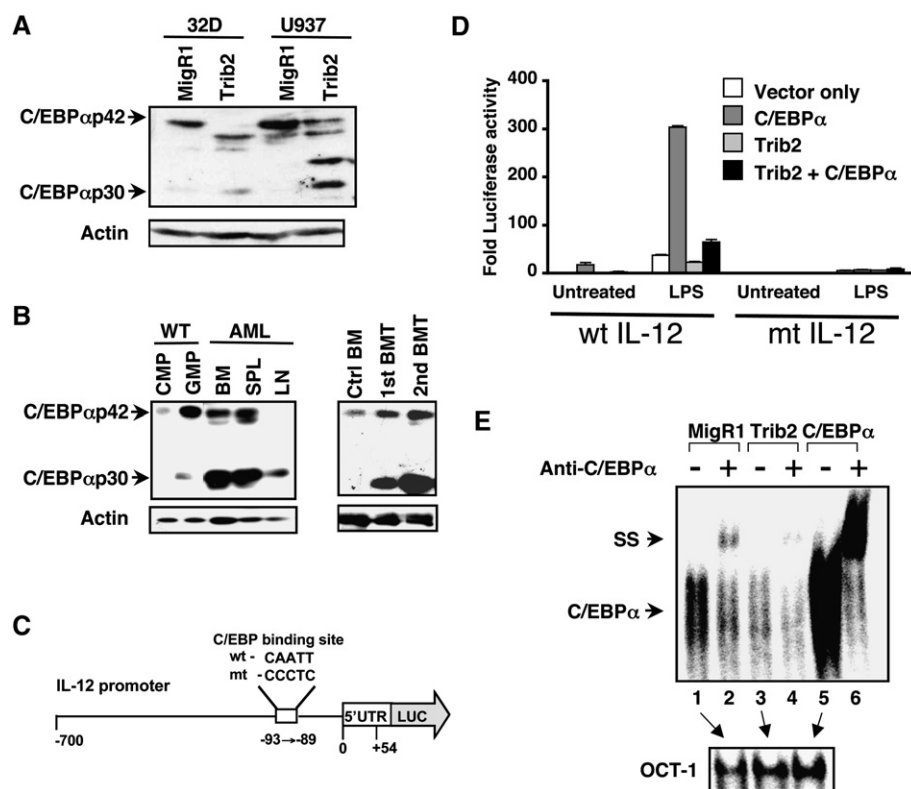


Figure 5. Trib2 reduces normal levels of C/EBP α and inhibits its activity

A: Sorted GFP $^{+}$ 32D and U937 cells transduced with either MigR1 or Trib2 were assessed for C/EBP α protein expression by western blot. C/EBP α p42 is the full-length protein, and C/EBP α p30 is the truncated protein. Actin is the protein loading control.

B: Analysis of C/EBP α p42 and p30 protein expression in primary leukemic samples from BM (93% GFP $^{+}$), spleen (63% GFP $^{+}$), and lymph node (88% GFP $^{+}$) compared to normal levels expressed in CMPs and GMPs from B6 BM (left panel). Levels of C/EBP α p42 and p30 proteins were also compared in unfractionated normal B6 mouse BM cells and primary (94% GFP $^{+}$) and secondary (98% GFP $^{+}$) leukemic BM samples (right panel).

C: Schematic of the IL-12 promoter containing the C/EBP α binding site.

D: RAW264.7 macrophages were cotransfected with (1) the IL-12 promoter firefly luciferase constructs containing the C/EBP wild-type or mutant binding sites; (2) either empty vector, Trib2, C/EBP α , or both Trib2 and C/EBP α ; and (3) a pRL-TK Renilla luciferase internal control plasmid. Luciferase activity was measured following LPS (100 ng/ml) treatment for 8 hr, 24 hr posttransfection. Reporter luciferase activity for each sample was normalized to the Renilla luciferase activity of the same sample. Data presented are mean \pm SD of triplicate cultures.

E: C/EBP α DNA binding activity was assessed by EMSA using a double-stranded oligonucleotide

probe containing the C/EBP binding site from the human G-CSF receptor. Nuclear extracts from sorted GFP $^{+}$ U937 cells transduced with MigR1 (lanes 1 and 2), Trib2 (lanes 3 and 4), or C/EBP α (lanes 5 and 6) were incubated with 32 P-labeled probe. In lanes 2, 4, and 6, 2 μ l of C/EBP α antibody was added. "SS" indicates the supershifted C/EBP α complex. The extracts used in lanes 1, 3, and 5 were tested with an OCT-1 probe in a second EMSA assay as a control for the integrity and quantity of nuclear binding proteins.

and 4). These data demonstrate that Trib2 expression inhibits the DNA binding function of C/EBP α p42.

As Trib2 overexpression decreased C/EBP α p42, we investigated whether siRNA-mediated knockdown of endogenous Trib2 would increase endogenous C/EBP α p42. Indeed, human U937 cells transfected with siRNA against human Trib2 had reduced Trib2 mRNA expression (Figure 6A) and a concomitant increase in C/EBP α p42 protein (Figure 6B). C/EBP α p30 is not detectable in these cells (see Figure 6D). Moreover, siRNA-mediated knockdown of retrovirally expressed murine Trib2 in U937 cells reduced murine Trib2 expression (Figure 6C) and abrogated its inhibitory effect on C/EBP α p42 (Figure 6D). Trib2 knockdown restored the C/EBP α p42:C/EBP α p30 ratio by increasing C/EBP α p42 expression. The failure to observe a concomitant decrease in C/EBP α p30 may stem from a long C/EBP α p30 half-life, residual Trib2 activity, or other unknown factors, and is a point that requires further investigation. Nevertheless, these data complement the Trib2 overexpression studies and further support the hypothesis that Trib2 opposes C/EBP α p42 function.

To further investigate the link between Trib2's inhibitory effects on granulopoiesis (Figure 1A) and C/EBP α p42 activity, we coexpressed Trib2 and C/EBP α p42 in 32D cells, which require IL-3 to survive and undergo C/EBP α p42-dependent granulocytic differentiation in response to G-CSF (Wang et al., 1999). To control C/EBP α p42 activity, we utilized an estradiol-inducible C/EBP α p42 fusion gene (C/EBP α ER) cloned in MigR1 (Camenga et al., 2003). In these experiments, Trib2 was expressed

in a MSCV retrovirus that also bears a tNGFR gene as a surrogate marker (Izon et al., 2001). 32D cells were sorted for retroviral expression of GFP (C/EBP α ER), tNGFR (Trib2), or both, treated with either estradiol or vehicle in the presence of either IL-3 or G-CSF, and then assayed for granulocytic differentiation by both flow cytometry (Figure 6E) and morphology (data not shown). As expected, vehicle-treated cells transduced with C/EBP α ER differentiated only in the presence of G-CSF (induction of endogenous C/EBP α activity), whereas estradiol (induction of exogenous C/EBP α activity) induced the differentiation of these cells in the presence of either G-CSF or IL-3 (Figure 6E). Further, cells transduced with Trib2 failed to differentiate in response to either G-CSF or IL-3, as did cells that were cotransduced with Trib2 and C/EBP α ER in the presence of vehicle. In contrast, the inhibitory effect of Trib2 in doubly transduced cells was overcome by estradiol treatment, indicating that high doses of C/EBP α overcome the inhibitory effects of Trib2.

Cell proliferation and survival were also assayed in these cultures by cell counting and trypan blue exclusion. 32D-C/EBP α ER cells proliferated in IL-3, and this proliferation was inhibited by either endogenous or exogenous C/EBP α activity (Figure S3, left bars). 32D-Trib2 cells proliferated in the presence of IL-3 but died in the presence of G-CSF (Figure S3, middle bars), an effect that was similar to transduction of 32D cells with a C/EBP α DNA binding mutant that was unable to induce differentiation (Keeshan et al., 2003). Cell numbers/viability were restored following estradiol induction of C/EBP α in the Trib2 + C/EBP α ER-expressing cells (Figure S3, compare two

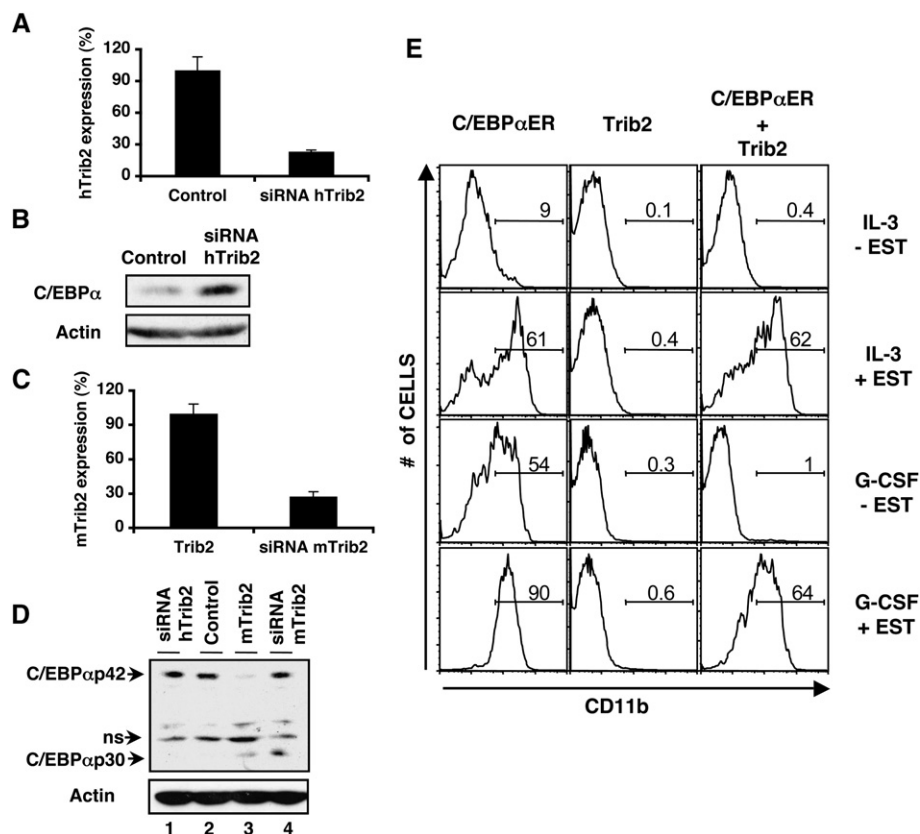


Figure 6. Trib2-dependent inhibition of C/EBPα expression and function is abrogated by siRNA-mediated knockdown of Trib2 and overexpression of C/EBPα.

A and B: siRNA designed to knock down human Trib2 (hTrib2) was electroporated into U937 cells and analyzed 24 hr later. **A:** hTrib2 expression assessed by real-time RT-PCR. Error bars denote ±SEM of each sample measured in triplicate. **B:** Western blot of C/EBPα protein expression. Lane 1, negative control siRNA; lane 2, hTrib2 siRNA. C/EBPαp30 is undetectable in parental U937 cells.

C and D: siRNA designed to knock down murine Trib2 (mTrib2) was electroporated into U937 cells retrovirally expressing murine Trib2 and analyzed 24 hr later. **C:** mTrib2 expression assessed by real-time RT-PCR. Error bars denote ±SEM of each sample measured in triplicate. **D:** Western blot of C/EBPαp42 and C/EBPαp30 protein expression. Lanes 1 and 2, U937 cells + human Trib2 siRNA and negative control siRNA, respectively, as in **B**; lanes 3 and 4, U937-mTrib2 cells + negative control siRNA and siRNA mTrib2, respectively. ns, nonspecific band.

E: 32D cells transduced with C/EBPαER (GFP), Trib2 (tNGFR), or C/EBPαER + Trib2 were sorted for GFP and/or tNGFR expression. Sorted cells were plated in equal numbers (1 × 10⁵) (day 0, 48 hr posttransduction) in IL-3 or G-CSF ± 1 μM β-estradiol (EST). CD11b expression was assessed after 2 days. Data are representative of three independent experiments.

far right bars). Similar to the CD11b expression data, these results also show that high doses of C/EBPα overcome Trib2's inhibitory effects.

We next sought to determine the mechanism by which Trib2 decreased normal C/EBPαp42 levels. In 32D and U937 cells, C/EBPαp42 levels in Trib2-expressing cells were restored by pretreatment with the proteasome inhibitor MG132 (Figure 7A). In coimmunoprecipitation experiments using 293T cells, Trib2 association with C/EBPαp42 was detected only in the presence of MG132, whereas C/EBPαp30 coimmunoprecipitated in the presence and absence of MG132 (Figure 7B, lane 4 and 5).

Expression of Trib2 in U937 cells decreased the half-life of endogenous C/EBPα protein as determined by ³⁵S metabolic pulse labeling and immunoprecipitation chase of labeled C/EBPα (Figure 7C). These data suggest that Trib2 acts by associating with C/EBPαp42 and promoting its degradation via the proteasome.

Elevated Trib2 expression is found in a subset of AML patients exhibiting C/EBPα defects

Since Trib2 is a potent inducer of AML in our experimental model, we investigated Trib2 expression in a mRNA expression

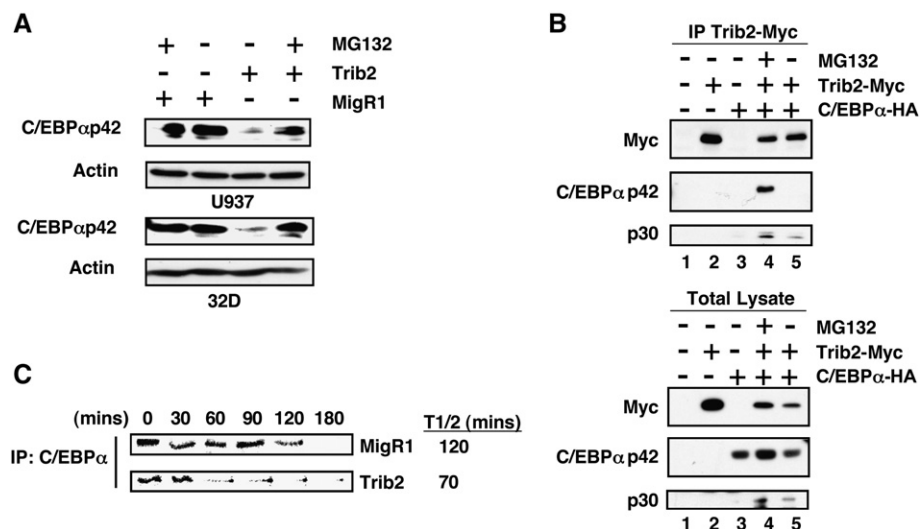


Figure 7. Trib2 forms a complex with C/EBPα and results in its proteasomal-dependent degradation.

A: Sorted GFP⁺ U937 (top panel) and 32D (lower panel) cells transduced with either MigR1 or Trib2, treated ± 10 μM MG132 for 2 hr, were assessed for C/EBPα expression by western blot.

B: 293T cells were transfected with empty vector (lane 1), myc-tagged Trib2 (lane 2), and HA-tagged C/EBPα (lane 3), or cotransfected with both (lanes 4 and 5), and treated with 10 μM MG132 for 2 hr (lane 4). Trib2 was immunoprecipitated using a Myc 9E10 antibody, and western blotting was performed with HA and Myc antibodies on immunoprecipitates (top panel) and total lysates (lower panel).

C: U937 cells transduced with MigR1 or Trib2 were metabolically labeled with ³⁵S-methionine for 60 min and chased for the indicated times. C/EBPα was immunoprecipitated, and radiolabeled protein was detected by SDS-PAGE. The half-life (T_{1/2}) was calculated using ImageJ software.

database derived from 285 AML patient samples. Previous analysis of gene expression in this data set identified 16 groups of AML with distinct gene expression profiles (Valk et al., 2004). Based on signals from two different *Trib2* probe sets, we noted that elevated *Trib2* expression was observed in cluster #4 (Figure 8), which is one of two clusters that are associated with *C/EBPα* mutations (Valk et al., 2004). Although cluster 4 contains AML samples with wild-type (green) or mutated (red) *C/EBPα*, *Trib2* expression was primarily elevated in tumors with wild-type *C/EBPα* (Figure 8, enlarged area). Thus, in an unbiased screen of AML samples, high levels of *Trib2* expression were preferentially found in a cohort associated with *C/EBPα* defects. This association again links *Trib2* to tumors with altered *C/EBPα* function and suggests that elevated *Trib2* may have a pathogenic role in a subset of human AML.

Discussion

Our data provide strong evidence that dysregulated *Trib2* expression contributes to the pathogenesis of AML. Retroviral expression of *Trib2* immortalized hematopoietic progenitors in vitro and induced fatal transplantable AMLs in murine recipients with a mean latency of 179 days. In accordance with the central role of *Trib2* in the development of these tumors, we have never observed AML in several hundred mice that have received MigR1-transduced BM cells using identical protocols. Microarray analysis of a cohort of primary human AMLs identified elevated *Trib2* mRNA expression in a cluster of tumors associated with a high frequency of *C/EBPα* mutations. Although we have not yet determined the mechanism of *Trib2* upregulation in these leukemias, our data linking *Trib2* expression to inhibition of *C/EBPα* function suggests that *Trib2* overexpression is likely to be an important pathogenic mechanism in a subset of human AML.

Decreased *C/EBPα*p42 levels are observed in diverse molecular subtypes of human AML (Helbling et al., 2004, 2005; Pabst et al., 2001a; Perrotti et al., 2002; Westendorf et al., 1998) and are sometimes associated with point mutations in *C/EBPα* (Pabst et al., 2001b). A number of observations suggest that *Trib2*-mediated alterations of *C/EBPα* function are likely to contribute to AML induction. We found that *Trib2* forms a complex with *C/EBPα*p42 that results in its destabilization through proteasome-dependent degradation. We further demonstrated an inverse functional relationship between *Trib2* and *C/EBPα* through both gain- and loss-of-function approaches. The effects of *C/EBPα* on myeloid cell differentiation are opposed by enforced *Trib2* expression, and we found high *Trib2* expression in a specific subset of human AMLs that is associated with *C/EBPα* mutations. We hypothesize that *Trib2*-expressing AMLs lacking *C/EBPα* mutations share a similar expression profile due to functional inactivation of *C/EBPα*. It is unlikely, however, that *Trib2* causes AML only through *C/EBPα* inactivation. Mice bearing knockout and mutated *C/EBPα* alleles have abnormalities of myeloid differentiation but do not develop AML (Heath et al., 2004; Zhang et al., 1997, 2004). Furthermore, despite its effect on *C/EBPα* function, *Trib2* is not sufficient for AML, as the tumors arising in *Trib2* mice were either mono- or bclonal.

In addition to *C/EBPα*, *Trib2* may influence other signaling pathways. In *Drosophila*, Tribbles degrades String/CDC25 to inhibit the cell cycle. Although it seems counterintuitive that decreased String/CDC25 would be oncogenic, antagonism of

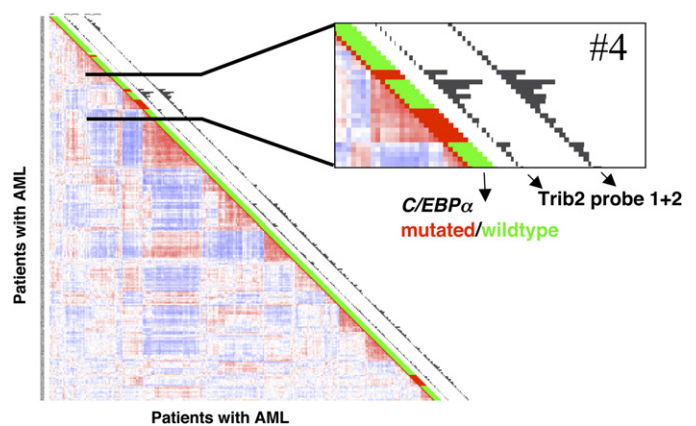


Figure 8. *Trib2* is elevated in a subset of human AML

Correlation view of 285 AML patients (Valk et al., 2004). Colors of cells relate to Pearson's correlation coefficient values: red indicates higher positive and blue indicates higher negative correlation between samples. Sixteen clusters represented by red blocks along the diagonal can be identified. *C/EBPα* mutation status is indicated next to each tumor (red, mutant; green, wild-type). Histograms next to each tumor represent expression levels of the two probe sets for *Trib2*. Cluster 4, one of the two clusters harboring most patients with *C/EBPα* mutations, has a significantly elevated expression of *Trib2* relative to other clusters.

String by Tribbles is associated with enhanced proliferation in certain contexts in the fly (Mata et al., 2000). Other Trib family members have been associated with Akt and MAPK signaling (Hegedus et al., 2006), and it remains to be determined whether *Trib2* influences these pathways in AML cells.

It is also possible that proviral integration by the *Trib2*-expressing retroviral vector created lesions in genes that co-operate with *Trib2*. We have begun to address this by cloning proviral integrations in *Trib2* tumors. In at least one *Trib2* AML, the provirus was found adjacent to *HoxA9*, whose mRNA was markedly elevated in this tumor (K.K., unpublished data). *HoxA9* has been shown to synergize with other oncogenes to accelerate AML development (Kawagoe and Grosveld, 2005). We are currently investigating the functional significance of this finding.

An additional property of *Trib2* is its association with increased *C/EBPα*p30 levels in cell lines and leukemic samples. *C/EBPα* is an intronless gene, and it has been suggested that the two major *C/EBPα* isoforms stem from alternative translational start sites. Other possibilities exist, however, as the homologous protein *C/EBPβ* is proteolytically processed into full-length and N-terminally truncated dominant-negative isoforms (Baer and Johnson, 2000; Lin et al., 1993; Ossipow et al., 1993; Welm et al., 1999). In our coimmunoprecipitation experiments, the *C/EBPα* expression construct lacked the uORF (upstream open reading frame) and contained a C-terminal HA tag that was detected with a HA antibody and verified with a specific *C/EBPα* antibody. This suggests that the 30 kDa protein is an N-terminal truncation of the full-length *C/EBPα* induced by *Trib2* expression. However, this construct retains the alternative ATG start codon, and therefore translational control cannot be ruled out. In favor of this possibility, we were unable to detect *C/EBPα*p30 in the pulse-chase experiment.

Although *Trib2* potentially induced AML in our BMT model, it is not clear whether the diminution of *C/EBPα*p42, the enhancement of *C/EBPα*p30, or both were critical to the phenotypes that we observed. Decreased *C/EBPα*p42 levels are associated

with human AML (Mueller and Pabst, 2006), and mice engineered to express a C/EBP α p42 mutant that fails to block the cell cycle develop a transplantable myeloproliferative disease that progresses to an AML-like phenotype but does not affect survival (Porse et al., 2005). Murine BM cells transduced with the dominant-negative C/EBP α p30 showed poor long-term engraftment (Schwieger et al., 2004). However, in CFU assays, the C/EBP α p30 dominant-negative was shown to block murine and human myeloid differentiation (Iwama et al., 2002; Schwieger et al., 2004; Wang and Friedman, 2002). Thus, both decreased C/EBP α p42 and increased C/EBP α p30 have been associated with myeloid transformation. Additional studies will be required to determine which of these isoforms is most relevant to Trib2-induced AML.

We initially identified *Trib2* by screening for transcripts that were downregulated when Notch1-dependent T cell tumor cell lines were induced to growth arrest by γ secretase inhibitor treatment (Weng et al., 2003). Although we find that Trib2 expression is modulated by Notch1 via CSL binding sites in the *Trib2* promoter (unpublished data), mice that retrovirally express activated Notch1 do not develop AML or exhibit major defects in granulocyte differentiation. Trib2 regulation by Notch1 may be context dependent and nonoperational in myeloid progenitors. However, Notch signaling promotes macrophage/DC differentiation in some assays (Cheng et al., 2003; Ohishi et al., 2001), and it will be interesting to determine if this is Trib2 dependent. In addition, the subset of human AMLs associated with increased *Trib2* expression are very immature and express T cell markers, such as *TCR δ* (Valk et al., 2004). Trib2 is highly expressed during several stages of T cell development (data not shown), and it is possible that Trib2 normally contributes to the expression of genes associated with T cell development.

Our data predict that Trib2 would functionally inactivate C/EBP α by causing its degradation. Thus, one expectation would be that it would associate with tumors in which C/EBP α is inactivated. Analysis of microarray data obtained from 285 AMLs showed that elevated *Trib2* expression preferentially associated with a cluster of AMLs characterized by C/EBP α defects. High *Trib2* expression was predominantly seen in tumors without C/EBP α mutations; however, it was also high in two tumors with these mutations (Figure 8). It may be that induction of this molecular class of AML requires C/EBP α function to be suppressed below a certain threshold, and that this may occur by mutation alone, Trib2 expression, or a combination of these two factors.

In summary, our studies identify Trib2 as an oncoprotein that contributes to the pathogenesis of AML through the inhibition of C/EBP α function. This occurs through the interaction of Trib2 with C/EBP α , resulting in the proteasomal-dependent degradation of C/EBP α . Our observations suggest that multiple leukemogenic mechanisms target C/EBP α in AML. Understanding the precise role of Trib2 in AML may lead to new diagnostic and therapeutic strategies for this aggressive human malignancy.

Experimental procedures

Constructs and retroviruses

A 1032 bp fragment encoding the entire murine *Trib2* cDNA was subcloned (\pm N terminus FLAG tag) into pcDNA3.1, pcDNA3.1/myc-HIS plasmid, or one of two different versions of MigR1 (one coexpressing GFP, the other truncated human nerve growth factor receptor [tNGFR]). C/EBP α rat cDNA (Keeshan et al., 2003) was cloned into the pcDNA3.1/myc-HIS plasmid. The MigR1-C/EBP α ER construct was a kind gift of Dr. S. Nimer. The IL-12 p40

promoter containing the B6 genomic fragment -700 to $+54$ of the IL-12 p40 gene was cloned into the pGL3-basic vector (Promega), and site-directed mutagenesis of the C/EBP binding site (-93 to -89) was performed using the QuickChange kit (Stratagene) according to the manufacturer's instructions (kind gifts from Dr. R Carmody).

32D differentiation assay

32D cells were transduced with C/EBP α ER (GFP), Trib2 (tNGFR + FLAG), or both, sorted for GFP and/or tNGFR 48 hr posttransduction, and cultured in DMEM without phenol red (GIBCO) containing 10% charcoal stripped FCS (Gemini Bioproducts) and 5 ng/ml rIL-3. Cells (1×10^5) were plated in 5 ng/ml IL-3 or 25 ng/ml G-CSF \pm 1 μ M β -estradiol (Sigma, E2257) and assessed for granulocytic differentiation by FACS analysis and morphological criteria.

Methylcellulose clonogenic assays

Sorted GFP $^+$ and GFP $^+$ /Lin $^-$ (CD3, CD4, CD8, B220, Gr-1, Ter119, CD19, MHCII, IL-7R α) BM cells from MigR1 and Trib2 transplanted mice were plated in triplicate in methylcellulose media (Methocult M3231, Stem Cell Technologies) supplemented with cytokines (10 ng/ml GM-CSF, 10 ng/ml IL-3, 10 ng/ml IL-6, 50 ng/ml SCF [Peprotech and BD Pharmingen]). Cells (15,000) from primary plates (colonies scored at 9 days) were transferred to secondary (colonies scored at 10 days) and tertiary (colonies scored at 8 days) plates containing the indicated cytokines, then transferred to RPMI liquid culture supplemented with 10% FBS and cytokines. Single colonies were transferred from primary plates to media containing IL-3, IL-6, and SCF in 24-well plates and assessed for growth after 11 days.

Bone marrow transduction and transplantation

C57BL/6 mice (B6) were obtained from Taconic Farms. Experiments were performed according to guidelines from the National Institutes of Health and with an approved protocol from the University of Pennsylvania Animal Care and Use Committee. Retroviral transduction of B6 BM cells and subsequent transfer of these cells into lethally irradiated recipients were performed as described (Pui et al., 1999), and details are provided in the [Supplemental Experimental Procedures](#). Seven independent transplant experiments were performed with different Trib2 retroviral constructs (MigR1-Trib2, MigR1-FLAG-Trib2, NGFR-FLAG-Trib2); each gave similar results and are summarized together in Figure 2A. Secondary transplants were performed by injecting 2×10^6 nucleated BM or spleen cells from the primary leukemic mice into sublethally irradiated (600 rads) B6 mice. Histology and immunohistochemical staining details are provided in the [Supplemental Experimental Procedures](#).

Flow cytometry

Cell suspensions were stained in PBS/2% FBS after blocking with nonspecific rat/mouse IgG (Sigma). Cells were sorted on a MoFlo (Cytomation) cell sorter. Analytical flow cytometry was performed on a FACS Calibur (Becton Dickinson) and analyzed using FlowJo software (Treestar). Antibodies used are described in the [Supplemental Experimental Procedures](#). Dead cells were excluded by a combination of forward and side scatter properties.

Immunoprecipitation, western blotting, and 35 S-methionine pulse-chase

293T cells were transfected with 3 μ g pcDNA3.1/myc-HIS-Trib2 and 2 μ g MigR1-C/EBP α . After 36 hr, cotransfected cells were treated with 10 μ M MG132 for 1 hr and 10 mM N-ethylmaleimide (both Calbiochem) for 30 s, washed in 1 \times PBS containing N-ethylmaleimide, and lysed with modified RIPA buffer (50 mM Tris [pH 8.0], containing 0.5% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na $_3$ VO $_4$, 1 mM NaF, and 20 mM N-ethylmaleimide) supplemented with protease inhibitors (Complete EDTA-free; Roche). Precleared lysates (3 mg) were incubated overnight with 10 μ l of protein A beads coated with 5 μ g anti-MYC 9E10 antibody. To detect C/EBP α in leukemic samples, cells were lysed directly in 2 \times SDS buffer. To detect Flag-tagged Trib2 in leukemic samples, protein lysates were prepared with modified RIPA buffer. Antibodies used were anti-C/EBP α (Sc-61, Santa Cruz), anti-HA (HA.11, Covance), anti-FLAG (M2,

Sigma), and anti-MYC (9E10). In pulse-chase experiments, U937 cells transduced with MigR1 and Trib2 were starved for 30 min, pulse-labeled for 60 min with ^{35}S -methionine (1 mCi/ml), and then chased for up to 180 min. Precleared protein lysates were incubated overnight with protein G beads coated with 5 μg C/EBP α antibody (SC-61x). Western blotting was performed according to standard procedures.

Luciferase reporter assay

RAW264.7 macrophages were transfected with various combinations of pcDNA control vector, vectors encoding Trib2 (390 ng) or C/EBP α (10 ng), IL-12 p40 (100 ng) wild-type and mutant promoter-luciferase (firefly) constructs, and the internal control Renilla-luciferase plasmid pRL-TK (10 ng; Promega). Firefly and Renilla luciferase activities were measured in whole-cell lysates using the Dual-Luciferase reporter assay kit (Promega), as per the manufacturer's instructions.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assays (EMSAs) were performed as described (Keshan et al., 2003), except that nuclear extracts were prepared using a Nuclear Extract Kit (Active Motif) as per the manufacturer's instructions. The G-CSF receptor promoter oligonucleotide (C/EBP site is underlined) had the sequence 5'-AAGGTGTTGCAATCCCCAGC-3'. The Oct-1 consensus oligonucleotide was obtained from Santa Cruz. Two microliters of C/EBP α (SC-61x) was used for supershift experiments.

Quantitative RT-PCR

RNA, isolated using the RNEasy kit (Qiagen), was digested with DNaseI and used for reverse transcription according to the manufacturer's instructions (Superscript II kit, Invitrogen). Validated human Trib2 and 18s rRNA primer/probe sets with TaqMan Universal PCR Master Mix (Applied Biosystems) and murine Trib2 primers (forward, 5'-AGCCGACTGTCTACAGAGA-3'; reverse, 5'-AGCGTCTTCCAACTCTCCA-3') with SYBR GREEN PCR Master mix (Applied Biosystems) were used for qRT-PCR and analyzed on the ABI Prism 7900 sequence detection system (Applied Biosystems).

DNA analysis

Southern blotting was performed according to standard procedures using QuikHyb (Stratagene) buffer and a ^{32}P -labeled IRES probe.

RNA interference

Human Trib2 siRNA (Silencer Validated siRNA #1060), murine Trib2 siRNA (Silencer Pre-designed siRNA #169360-exon2), and control Silencer Negative Control #1 were obtained from Ambion. U937 parental cells (2×10^6) (expressing human Trib2) and U937 cells that were transduced with Trib2 (murine) in 100 μl of Amaxa solution (Nucleofector Kit V, Amaxa Biosystems) were mixed with 100 nM of siRNA and electroporated using the Amaxa Nucleofector. Trib2 (human and murine) mRNA expression and C/EBP α protein expression were assessed 24 hr later.

Supplemental data

The Supplemental Data include Supplemental Experimental Procedures, three supplemental figures, and two supplemental tables and can be found with this article online at <http://www.cancer.org/cgi/content/full/10/5/401/DC1/>.

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