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LEF-1 is crucial for neutrophil granulocytopoiesis and its expression is severely reduced in congenital neutropenia

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We demonstrate here that lymphoid enhancer-binding factor 1 (LEF-1) mediates the proliferation, survival and differentiation of granulocyte progenitor cells. We initially documented the importance of this transcription factor in the bone marrow of individuals with severe congenital neutropenia (CN) with a 'differentiation block' at the promyelocytic stage of myelopoiesis[1]. LEF-1 expression was greatly reduced or even absent in CN arrested promyelocytes, resulting in defective expression of the LEF-1 target genes *CCND1*, *MYC* and *BIRC5*, encoding cyclin D1 (ref. 2), c-Myc[3] and survivin[4], respectively. In contrast, healthy individuals showed highest LEF-1 expression in promyelocytes. Reconstitution of LEF-1 in early hematopoietic progenitors of two individuals with CN corrected the defective myelopoiesis and resulted in the differentiation of these progenitors into mature granulocytes. Repression of endogenous LEF-1 by specific short hairpin RNA inhibited proliferation and induced apoptosis of CD34$^+$ progenitors from healthy individuals and of cells from two myeloid lines (HL-60 and K562). C/EBPα, a key transcription factor in granulopoiesis[5,6], was directly regulated by LEF-1. These observations indicate that LEF-1 is an instructive factor regulating neutrophilic granulopoiesis whose absence plays a critical role in the defective maturation program of myeloid progenitors in individuals with CN.

Severe congenital neutropenia (CN) is a rare disorder of myelopoiesis resulting in recurrent life-threatening infections due to a lack of peripheral blood neutrophils[1]. CN is also considered a pre-leukemic syndrome, because more than 20% of patients with CN progress to acute myelogenous leukemia (AML)[7]. All individuals with CN have a characteristic bone marrow phenotype that distinguishes the condition from other neutropenias: 'maturation arrest' with accumulation of granulocyte precursors (promyelocytes) and absence of mature granulocytes. The arrested CN promyelocytes show impaired proliferation and differentiation in response to granulocyte colony-stimulating factor (G-CSF), as well as accelerated apoptosis[8,9]. The characteristic block in promyelocyte differentiation in CN provides an excellent model for investigating the regulation of myelopoiesis in humans.

CN has been proposed to be a heterogeneous disorder involving mutations in various genes, including those encoding neutrophil elastase (ELA2), G-CSF receptor (G-CSFR), GFI-1 and WASP[10–13]. Because there are also many cases of CN not associated with any known mutations, the definitive common pathologic mechanism of this syndrome remains to be identified. Analysis of transcription factors may help to delineate the molecular mechanisms of CN. Factors relevant to CN pathology should be specifically affected only in CN but not in other neutropenias, and their restoration to normal levels should overcome the maturation arrest.

LEF/TCFs (or T-cell factors) are a family of transcription factors regulated by the canonical Wnt signaling pathway and generally act in transcriptional complexes with β-catenin[14]. LEF-1 is also known to act independently of β-catenin (for example, in the TGF-β and Notch pathways)[15,16]. In addition, recent studies have described a dominant-negative LEF-1 isoform (dnLEF-1) that lacks the β-catenin binding domain and functions as either a transcriptional repressor or transcriptional activator[17,18]. To date, analysis of the role of LEF-1 in hematopoiesis has been mostly restricted to the lymphoid compartment[14,19–22].

To study the regulation of myelopoiesis, we initially characterized mRNA expression patterns of different transcription factors in CD33$^+$ myeloid progenitors (which consist predominantly of promyelocytes) from individuals with CN in comparison to healthy controls. We found that LEF-1 mRNA expression was 20-fold downregulated, or even completely absent, in CN-derived samples. We confirmed down-regulation of LEF-1 mRNA in CN-derived bone marrow cells at different stages of myeloid differentiation, which were isolated by laser-assisted single cell picking. In healthy controls, we found varying LEF-1 expression levels at all stages of myelopoiesis, with peak expression in promyelocytes. LEF-1 mRNA expression in promyelocytes of individuals with CN was significantly lower ($P < 0.05$) than in healthy controls and in some cases was absent (**Fig. 1a**). We confirmed this on the protein level by observing a lack of fluorescent

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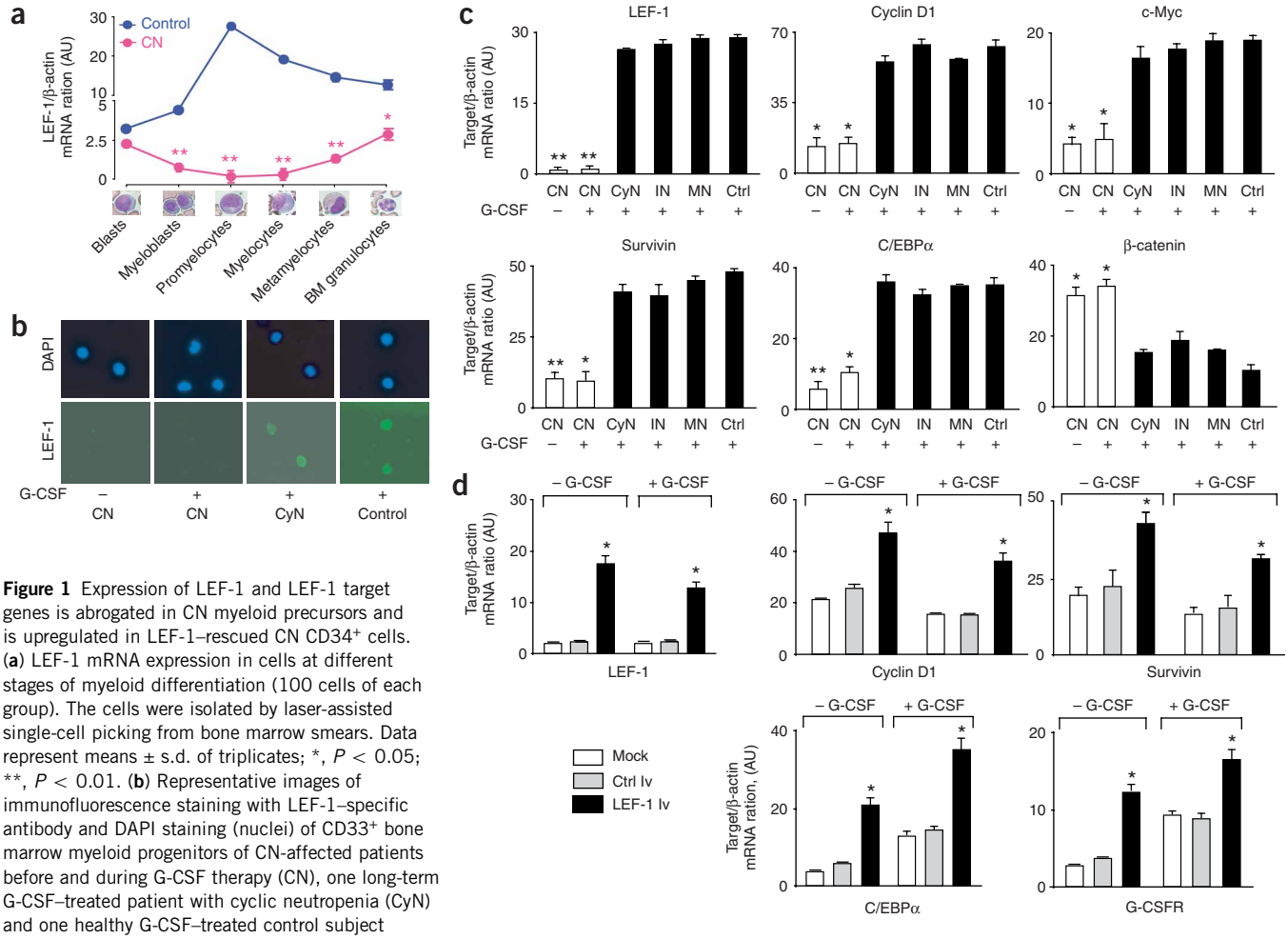

Figure 1 Expression of LEF-1 and LEF-1 target genes is abrogated in CN myeloid precursors and is upregulated in LEF-1–rescued CN CD34$^+$ cells. (**a**) LEF-1 mRNA expression in cells at different stages of myeloid differentiation (100 cells of each group). The cells were isolated by laser-assisted single-cell picking from bone marrow smears. Data represent means ± s.d. of triplicates; *, $P < 0.05$; **, $P < 0.01$. (**b**) Representative images of immunofluorescence staining with LEF-1–specific antibody and DAPI staining (nuclei) of CD33$^+$ bone marrow myeloid progenitors of CN-affected patients before and during G-CSF therapy (CN), one long-term G-CSF–treated patient with cyclic neutropenia (CyN) and one healthy G-CSF–treated control subject (Control). *, $P < 0.05$; **, $P < 0.01$. (**c**) mRNA expression in CD33$^+$ cells of studied groups, as described in Methods. IN, idiopathic neutropenia; MN, neutropenia associated with congenital disorders of metabolism (see Methods); Ctrl, control. Data represent means ± s.d. and were measured in duplicate; *, $P < 0.05$; **, $P < 0.01$ compared to Ctrl. (**d**) mRNA expression in LEF-1 lentivirus (lv)–, control (ctrl) lv–, or mock–transduced CN CD34$^+$ cells ($n = 2$) measured on day 4 of culture with or without G-CSF. Data represent means ± s.d. and cells were transduced and measured in triplicate; * $P < 0.05$ compared to Ctrl lv. AU, arbitrary units.

signal only in CN CD33$^+$ myeloid progenitors stained with a LEF-1–specific antibody (**Fig. 1b** and **Supplementary Fig. 1** online). To investigate whether LEF-1 downregulation was specific to the granulocytic lineage, we tested LEF-1 expression in CD14$^+$ monocytes and CD3$^+$ T-lymphocytes. In these cell populations, LEF-1 expression levels in individuals with CN were nearly identical to those in healthy individuals (**Supplementary Fig. 1**). This pointed to a lineage-specific reduction of LEF-1 mRNA and protein in CN granulocyte precursors.

To analyze whether the absence of LEF-1 is a distinct feature of CN and not common in other types of neutropenia, we studied CD33$^+$ cells from individuals with cyclic neutropenia, idiopathic neutropenia and neutropenias associated with congenital disorders of metabolism. We found significantly decreased ($P < 0.05$) expression of LEF-1 and of cyclin D1, survivin and c-Myc (all encoded by LEF-1 target genes), as well as of the key granulopoietic transcription factor C/EBPα, only in individuals with CN (**Fig. 1c** and **Supplementary Fig. 1**). In contrast, the mRNA expression profiles of two other LEF-1 family members, TCF-3 and TCF-4, were similar in all neutropenias. Notably, expression of β-catenin, the LEF-1 binding partner in the canonical Wnt pathway, was twofold higher in individuals with CN than in the other groups studied.

Pharmacological doses of G-CSF (1–100 μg per kg body weight per day) are clinically effective in overcoming the 'maturation arrest' of promyelocytes in patients with CN[1]. Therefore, we compared the expression of the aforementioned genes in subjects with CN before and after G-CSF therapy. Notably, long-term G-CSF treatment had no effect on LEF-1 mRNA and protein expression in individuals with CN (**Fig. 1b**,**c**).

To investigate whether downregulation of LEF-1 is caused by mutations in the LEF-1 promoter, we performed sequence analysis of the previously characterized 2,700-bp LEF-1 promoter[23]. We found no mutations in any of the groups studied (data not shown), which points to a post-transcriptional regulatory defect.

To address the significance of the absence of LEF-1 in CN-affected individuals, we re-expressed LEF-1 in CD34$^+$ cells of two subjects with CN using lentiviral-based constructs containing a *LEF1* cDNA (LEF-1 lv). This resulted in marked upregulation of mRNA expression of LEF-1 and its target genes, as well as of the genes encoding C/EBPα and G-CSFR, as compared to control groups (**Fig. 1d** and **Supplementary Fig. 2** online). As G-CSF induces C/EBPα, the LEF-1–dependent increase of C/EBPα expression was surprising. Notably, mRNA expression levels of C/EBPα and G-CSFR in LEF-1–transduced

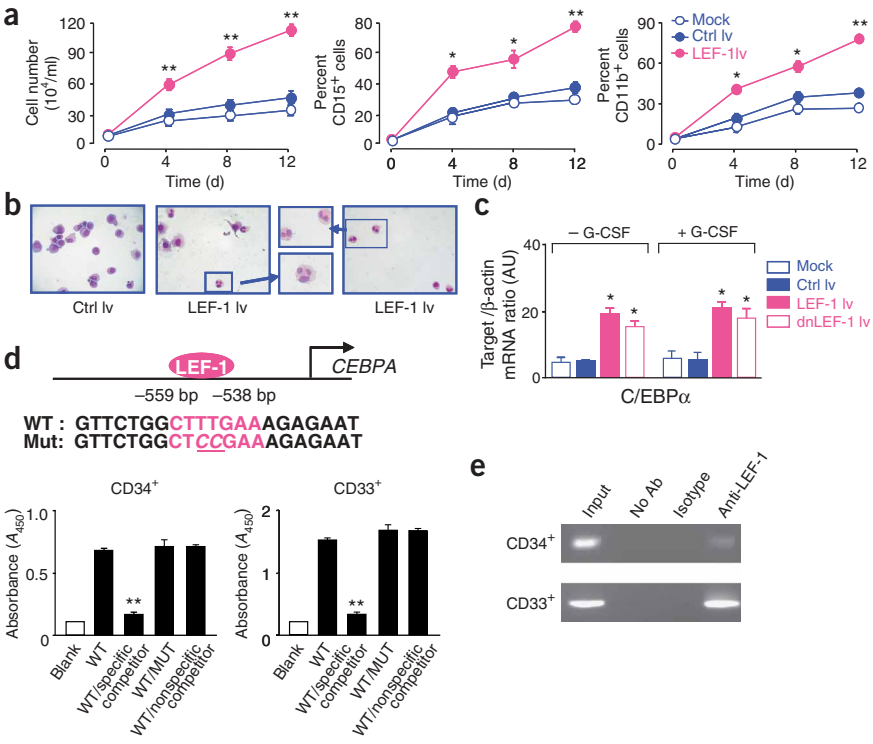

Figure 2 Restoration of defective LEF-1 expression promotes granulocytic differentiation of CN CD34$^+$ progenitors. LEF-1 induces C/EBPα expression through direct binding to the C/EBPα promoter. (**a**) Cell numbers and granulocytic (CD15) and myeloid (CD11b) surface marker expression after culture with G-CSF of CD34$^+$ CN cells transduced with the indicated constructs ($n = 2$). Viable cell numbers were measured by trypan blue dye exclusion and cell surface marker expression was measured by FACS. (**b**) Wright-Giemsa staining of LEF-1 lv– or Ctrl lv–transduced CD34$^+$ CN cells on day 12 of differentiation. (**c**) C/EBPα mRNA expression of CN CD34$^+$ cells transduced with the indicated constructs ($n = 2$) measured after 12 h of culture. AU, arbitrary units. Data represent means ± s.d. and derive from three experiments each done in triplicate; *, $P < 0.05$; **, $P < 0.01$. (**d**) The LEF-1 binding site (CTTTGAA; depicted in magenta) was mapped to the region between –559 bp to –538 bp of the 566 bp *CEBPA* promoter using a NoShift competitor assay, which measured LEF1 DNA-binding activity in nuclear extracts (NE) of CD34$^+$ and CD33$^+$ bone marrow cells. Biotinylated oligonucleotides corresponding to the wild type sequence shown were used in the assay (WT), or used together with nonbiotinylated WT oligonucleotides as a specific competitor (WT/specific competitor); nonbiotinylated oligonucleotides with a mutant LEF1 consensus-binding motif (WT/Mut); or nonbiotinylated oligonucleotides without a LEF1 consensus sequence as a nonspecific competitor (WT/nonspecific competitor) ; **, $P < 0.01$, compared to WT (**e**) ChIP assay of NE from CD34$^+$ and CD33$^+$ bone marrow cells; PCR products were amplified using primer pairs flanking the LEF1 binding site of the *CEBPA* promoter. No Ab, no antibody; isotype, isotype antibody control.

cells were further increased in G-CSF–treated cells. Transduction of CD34$^+$ cells with LEF-1 increased G-CSF–induced terminal granulocytic differentiation, whereas mock– or empty virus–transduced cells remained defective in granulopoiesis (**Fig. 2a**,**b**). Indeed, LEF-1 was able to overcome the typical maturation block normally evident in CN progenitors. LEF-1–dependent but G-CSF–independent regulation of C/EBPα was confirmed by the early increase in C/EBPα mRNA expression observed in LEF-1 transduced CD34$^+$ CN cells (after 12 and 24 h of culture) (**Fig. 2b**, **Supplementary Fig. 2** and data not shown). Notably, transduction of cells with dnLEF-1, which lacks the β-catenin–binding domain, resulted in upregulation of C/EBPα to a degree similar to that observed with full-length LEF-1 (**Fig. 2c**). This demonstrates that LEF-1 regulates C/EBPα independently of β-catenin.

A screen of the previously characterized 566-bp upstream promoter of *CEBPA*[24], the gene encoding C/EBPα, revealed a putative LEF-1 binding site (–559 bp to –538 bp). We confirmed LEF-1 binding in nuclear extracts from CD34$^+$ and CD33$^+$ cells. In the NoShift assay, the intact consensus LEF-1 binding site of the biotinylated DNA probe was required for the binding, as shown in a competition assay with non-biotinylated LEF-1–specific, LEF-1–nonspecific and mutated LEF-1–specific probes (**Fig. 2d**). Specificity of the LEF-1 binding to the *CEBPA* promoter was also indicated in a chromatin immunoprecipitation (ChIP) assay by the presence of the specific band in the anti–LEF-1 precipitate and by the absence of amplicons in isotype controls (**Fig. 2e**). LEF-1 bound to the *CEBPA* promoter more efficiently in CD33$^+$ myeloid progenitors as compared to CD34$^+$ cells (**Fig. 2d**,**e**). Together with the results presented above showing LEF-1–dependent C/EBPα expression, these data clearly indicate that LEF-1 directly regulates C/EBPα.

C/EBPα is well known to have a crucial role in regulating the balance between proliferation and differentiation of myeloid precursors and to be a key factor in the induction of granulocyte differentiation[5,6,25–27]. Targeted disruption of *CEBPA* causes a selective block in granulocytic differentiation, an observation that supports the role of C/EBPα in this process[28]. Our data suggest that C/EBPα is a LEF-1–dependent differentiation factor and that LEF-1–dependent downregulation of C/EBPα expression in individuals with CN (a pre-leukemic syndrome)[7] leads to a maturation block in promyelocytes similar to that which has been reported for dominant-negative C/EBPα mutations in AML[29,30]. In contrast to AML, where *CEBPA* is mutated and therefore leads to a non-functional C/EBPα product, C/EBPα expression is downregulated in individuals with CN as a result of LEF-1 dysfunction.

We then investigated the role of LEF-1 in cell proliferation and survival. We inhibited LEF-1 expression in CD34$^+$ cells from healthy individuals using LEF-1–specific short hairpin (sh) RNA. We compared the phenotype of LEF-1 shRNA–transduced cells with that of cells in which β-catenin was downregulated as well as with that of control samples. Upon downregulation of LEF-1 expression, we observed a significant decrease in mRNA expression of its target genes ($P < 0.05$), as compared to controls (**Fig. 3a**,**b** and **Supplementary Fig. 3** online). Moreover, we found a threefold reduction in the proportion of proliferating cells after LEF-1 knockdown (**Fig. 3c**). There was no increase in the number of viable cells after LEF-1 inhibition, as 57% of cells were apoptotic (**Fig. 3d**,**e** and **Supplementary Fig. 3**). This demonstrates the importance of LEF-1 not only for granulocytic differentiation but also for the proliferation and survival of CD34$^+$ cells. Notably, LEF-1 inhibition in CD34$^+$ cells had no effect on GM-CSF–triggered differentiation toward monocytes/macrophages

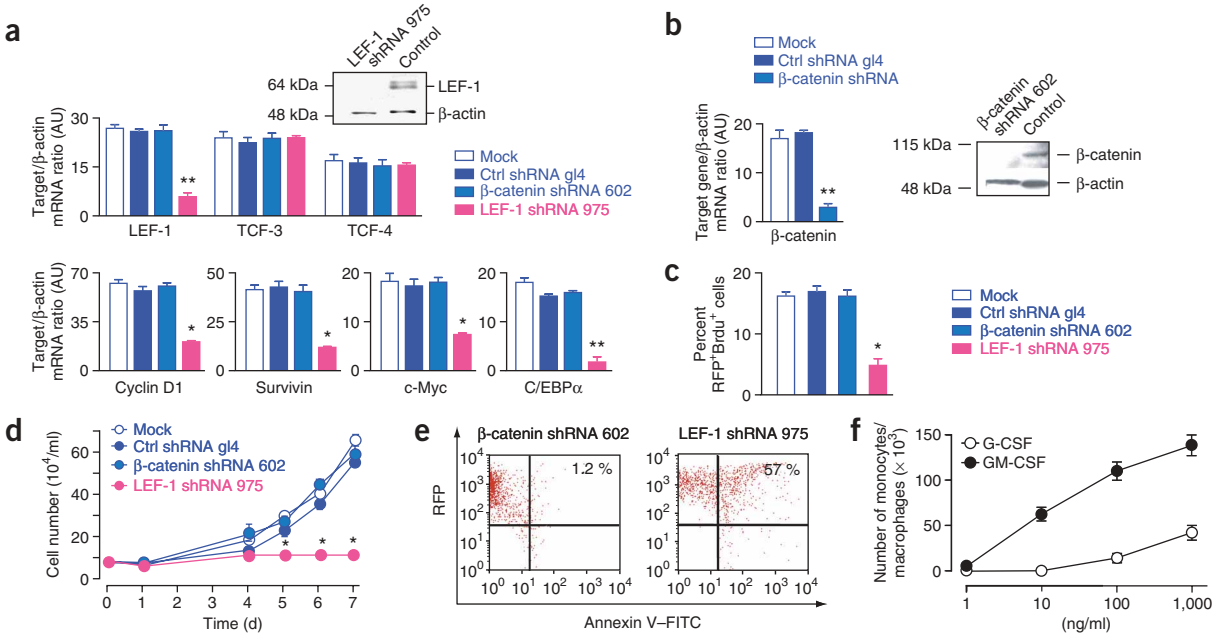

Figure 3 Anti-proliferative and pro-apoptotic effects of LEF-1 inhibition in CD34$^+$ progenitors. CD34$^+$ cells from healthy individuals ($n = 3$) were transduced with LEF-1 shRNA (LEF-1 shRNA 975), control shRNA (ctrl shRNA gl4) or β-catenin shRNA (β-catenin shRNA 602) all containing an RFP reporter. (**a**) mRNA expression of indicated genes in transduced cells measured on day 4 after transduction. Inset: western blot analysis of LEF-1 protein expression in total lysates of CD34$^+$ cells, transduced with control or LEF-1 shRNA. (**b**) β-catenin mRNA and protein expression in CD34$^+$ cells transduced with control or β-catenin shRNA. (**c**) Proliferation of sorted RFP$^+$ cells by counting RFP$^+$BrdU$^+$ double-positive cells by FACS on day 4 after transduction. (**d**) Viable cells were counted using trypan blue dye exclusion. (**e**) Percentage of apoptotic cells (annexin V–FITC$^+$ RFP$^+$) on day 4 after transduction. (**f**) Monocyte/macrophage numbers obtained upon treatment of LEF-1 shRNA–transduced, sorted CD34$^+$ cells (10^3 cells per well) with 10, 100 or 1,000 ng/ml of GM-CSF or G-CSF, as determined by counting on Wright–Giemsa-stained cytospin slides on day 7 of culture. In **a**–**d**, **f**, data represent means ± s.d. and cells were transduced and measured in duplicate; *, $P < 0.05$; **, $P < 0.01$.

(**Fig. 3f**), suggesting a specific role for LEF-1 in the granulocytic but not the monocytic lineage.

In two myeloid leukemia cell lines, HL-60 and K562, LEF-1 inhibition resulted in downregulation of target genes, similar to that seen in LEF-1 shRNA–transduced CD34$^+$ cells from healthy individuals. In addition, we observed strongly enhanced apoptosis and a block in cell proliferation in both cell lines (**Supplementary Figs. 4** and **5** online). Downregulation of β-catenin in K562 myeloid cells did not alter cell proliferation, apoptosis or LEF-1 target gene expression (**Supplementary Figs. 4** and **5**). Because β-catenin protein expression was marginal in HL-60 cells, anti–β-catenin shRNA in these cells was not included in this study.

The importance of LEF-1 for survival and proliferation of CD34$^+$ cells prompted us to ask whether LEF-1 overexpression could increase their proliferation. Indeed, LEF1 overexpression resulted in enhanced cell proliferation and upregulation of LEF-1 target genes as well as G-CSFR mRNA levels (**Fig. 4a–d** and **Supplementary Fig. 6** online). Mock-tranduced or empty virus–transduced CD34$^+$ cells only gradually increased in cell number, to a maximum of approximately tenfold, until day 12, and then declined.

Previous reports describing the role of LEF-1 in hematopoietic cells have been restricted mostly to lymphoid tissues. In accord with our findings in myelopoiesis, others have demonstrated that LEF-1 regulates proliferation and survival of lymphoid progenitors, namely pro-B cells, pre-B cells and early thymic progenitors[14,19–22]. From a broader perspective, LEF-1 is important at a particular precursor stage of both lymphopoiesis and granulopoiesis (**Fig. 4e**). In myelopoiesis this seems to be under cytokine control, as a link between cytokine response and LEF-1 function is evident: CD34$^+$ progenitors from two individuals with CN showed defective granulopoiesis even in the presence of G-CSF, and this defect was corrected by reconstitution of LEF-1 expression in these cells. Physiological concentrations of G-CSF did not increase C/EBPα expression in individuals with CN (**Fig. 1b,c**). However, pharmacological doses of G-CSF *in vitro*, such as we used in clinical trials (100–1,000 times greater than physiological levels)[1], moderately upregulated C/EBPα independently of LEF-1 *in vitro* (**Fig. 2a**). This could explain why individuals with CN respond to pharmacological doses of G-CSF *in vivo*.

GM-CSF has no effect on neutrophil generation in CN-affected individuals, but only increases the number of monocytes and eosinophils[31]. *In vitro*, CD34$^+$ cells from healthy individuals treated with LEF-1 shRNA responded to GM-CSF by differentiating toward monocytes/macrophages. Therefore, LEF-1 is not required for monopoiesis.

Our search for a common pathologic mechanism of CN led us to identify LEF-1 as an important transcription factor regulating the differentiation of myeloid progenitors to mature neutrophils: LEF-1 is highly expressed in normal promyelocytes but its expression is greatly reduced in the 'arrested' promyelocytes seen in CN patients. The differentiation defect of CN promyelocytes could be due to the loss of LEF-1 target gene expression. One prominent target is C/EBPα, which seems likely to control promyelocyte differentiation: C/EBPα is known to be important for neutrophil lineage-specific differentiation[25–27] and we observed that its expression is downregulated in CN. In addition, LEF-1 inhibition resulted in reduced proliferation and increased apoptosis of CD34$^+$ progenitors. These effects are most likely caused by downregulation of cyclin D1, c-Myc and survivin, such as is observed in patients with CN. C/EBPα can also have anti-proliferative effects in certain circumstances, although we observed only a net

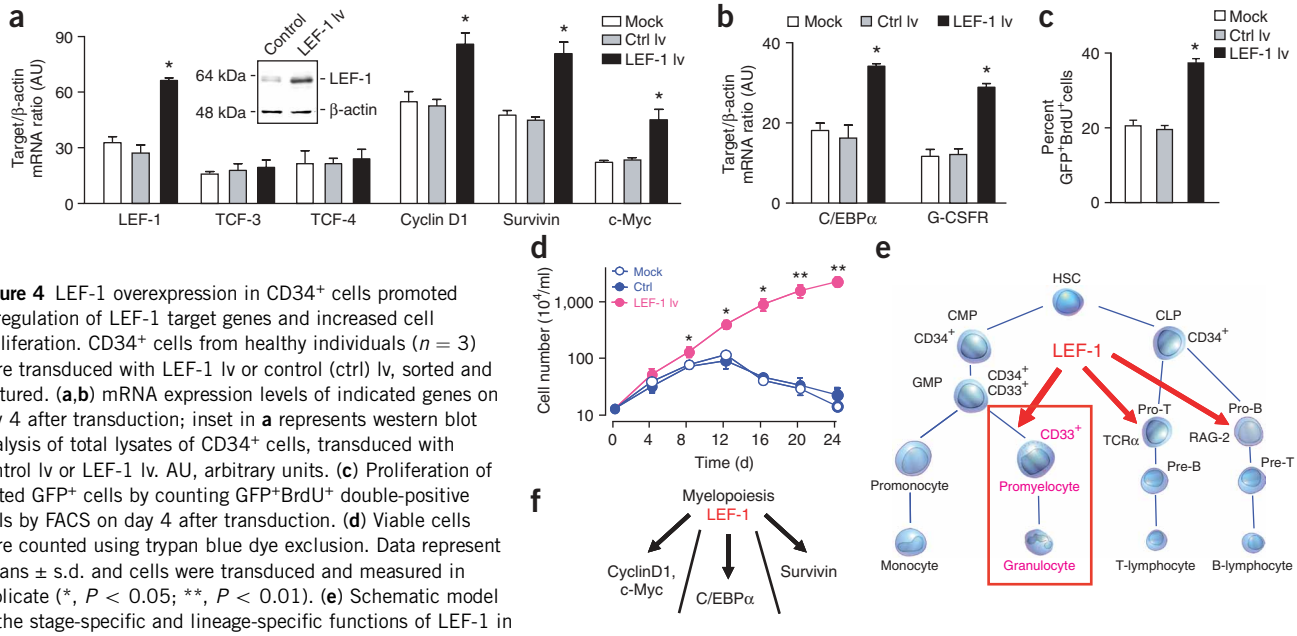

Figure 4 LEF-1 overexpression in CD34$^+$ cells promoted upregulation of LEF-1 target genes and increased cell proliferation. CD34$^+$ cells from healthy individuals ($n = 3$) were transduced with LEF-1 lv or control (ctrl) lv, sorted and cultured. (**a**,**b**) mRNA expression levels of indicated genes on day 4 after transduction; inset in **a** represents western blot analysis of total lysates of CD34$^+$ cells, transduced with control lv or LEF-1 lv. AU, arbitrary units. (**c**) Proliferation of sorted GFP$^+$ cells by counting GFP$^+$BrdU$^+$ double-positive cells by FACS on day 4 after transduction. (**d**) Viable cells were counted using trypan blue dye exclusion. Data represent means ± s.d. and cells were transduced and measured in triplicate (*, $P < 0.05$; **, $P < 0.01$). (**e**) Schematic model of the stage-specific and lineage-specific functions of LEF-1 in hematopoiesis. LEF-1 induces granulocytic differentiation of promyelocytes (red box), similar to its effects in lymphocyte differentiation at the pre-mature stage (red arrows). (**f**) Effects of LEF-1 on the initiation of the myelopoietic maturation program are mediated through the regulation of distinct target genes.

positive effect on the proliferation of CD34$^+$ cells transduced with C/EBPα shRNA (J.S. and M.S., unpublished data). We found that the levels of cyclin D1 and survivin mRNA remained unchanged after C/EBPα inhibition, and c-Myc expression increased slightly (J.S. and M.S., unpublished data). Therefore, we propose that downregulation of cyclin D1 results in a stronger signal for growth inhibition than a potentially positive effect of C/EBPα on proliferation. C/EBPα-mediated growth arrest occurs through protein-protein interactions (for example, with p21 or E2F) and is independent of its DNA binding and transcriptional activity, whereas the induction of differentiation requires DNA binding[32]. Thus, only the transcriptional activities of C/EBPα that induce differentiation might be directly dependent on LEF-1. Previous studies have shown an inverse relation of c-Myc expression to C/EBPα[33] expression, which points to a context-dependent, intertwined regulation of differentiation and proliferation downstream of LEF-1.

The absence of LEF-1 results in a distinctive phenotype in CD34$^+$ cells and in CN, even though the expression of other TCFs (TCF-3 and TCF-4) is normal. This may be due to certain differences in their structures: only LEF-1 contains a context-dependent activating domain that interacts with the transcriptional coactivator ALY, whereas TCF-3 and TCF-4 contain a binding domain for C-terminal binding protein (CtBP)[34]. It is known that LEF-1/TCF proteins are nonredundantly required for proper mesoderm induction in *Xenopus laevis*[35] and maintenance of skin stem cells in mice[12]. These data, in conjunction with our findings, clearly argue against redundant functions of these factors. LEF-1/TCFs may each regulate different genes and be active in different stages of cell proliferation and differentiation. In addition, we observed that rescue of CN progenitors with either full-length LEF-1 or dnLEF-1 resulted in upregulation of C/EBPα, one of the LEF-1 target genes and β-catenin inhibition caused no phenotypic differences in the CD34$^+$ cells of healthy individuals (**Fig. 3a–e** and **Supplementary Fig. 3**). Therefore, we propose that LEF-1 regulates myelopoiesis in a β-catenin–independent manner, similar to what occurs in LEF-1 regulation of T-lymphocyte development[21].

Because CN is a heterogeneous syndrome and causative mutations have not been identified in many individuals with CN, the common pathologic mechanism of this syndrome is unclear. We have shown here that when LEF-1 is absent, as is the case in CN, a maturation arrest of myeloid progenitors occurs, and we believe that this molecular mechanism is common and specific for CN. Forty-six percent of the CN-affected subjects studied here carried a mutation in *ELA2* (**Supplementary Table 1** online), but we observed no correlation between these mutations and LEF-1 expression: little or no LEF-1 was detectable irrespective of *ELA2* mutation status. We detected no mutations affecting the genes encoding G-CSFR, WASP or GFI-1 in the CN-affected subjects studied here.

In summary, we propose that the CD34$^+$ differentiation program toward mature neutrophils is regulated by LEF-1 through two distinct mechanisms: (i) by upregulation of expression of proliferative and anti-apoptotic proteins such as cyclin D1, c-Myc and survivin and (ii) by control of proper lineage commitment and granulocytic differentiation through regulation of C/EBPα (**Fig. 4f**).

METHODS

Affected and control subjects. Participants in this study included 13 patients with CN, 4 with cyclic neutropenia, 4 with neutropenias associated with congenital disorders of metabolism (1 with glycogen storage disease type Ib and 3 with Shwachman-Diamond syndrome) and 2 with idiopathic neutropenia (all of these subjects had received long-term (>1 year) G-CSF treatment; G-CSF dose ranged between 1.2 and 7.5 μg per kg body weight per day, or once in 2 d). Two CN patients had not yet received G-CSF therapy, and three healthy volunteers received G-CSF at a dose of 5 μg per kg body weight per day for 2 d. We collected bone marrow samples in association with the annual follow-up recommended by the Severe Chronic Neutropenia International Registry (SCNIR). Informed consent was obtained from all subjects. We obtained approval for this study from the Hannover Medical School's institutional review board.

Cell purification and separation. We isolated bone marrow and blood mononuclear cells by Ficoll-Hypaque gradient centrifugation (Amersham Biosciences) and positively selected bone marrow CD34$^+$CD33$^+$ and blood

$CD14^+CD3^+$ cells by sequential immunomagnetic labeling with corresponding MACS beads (Miltenyi Biotech). For shRNA experiments, we used G-CSF–primed peripheral blood $CD34^+$ cells.

Quantitative real-time RT-PCR (qRT-PCR). For qRT-PCR, we isolated RNA using the Qiagen RNeasy Mini Kit (Qiagen) or Trizol reagent (Invitrogen) using manufacturers' protocols with slight modifications (**Supplementary Methods** online), amplified cDNA using random hexamer primer (Fermentas) and measured mRNA expression using the SYBR green qPCR kit (Qiagen). Target gene mRNA expression was normalized to *ACTB* (encoding β-actin) and is represented as arbitrary units. Primer sequences are available upon request. *LEF1* mRNA primers detected both full-length and dn *LEF1*.

Laser-assisted cell picking. We isolated cells from bone marrow slides using the PALM Laser-MicroBeam System (P.A.L.M.) and controlled the purity of individual populations (100 cells per sample) by qRT-PCR of transcripts encoding myeloid-specific primary (myeloperoxidase; MPO) and secondary (matrix metalloproteinase-9; MMP9) granule proteins (**Supplementary Fig. 1**).

Confocal fluorescence microscopy. We fixed 1×10^5 $CD33^+$ cells on slides in 4% paraformaldehyde for 10 min, permeabilized them with 0.5% Triton X-100 for 20 min, and incubated them with LEF-1–specific rabbit polyclonal antibody (1:2,000 dilution) for 1 h at 37 °C and then with secondary FITC-conjugated antibodies for 30 min. Nuclei were counterstained with DAPI.

Analysis of LEF-1 binding to the *CEBPA* promoter in nuclear extracts of $CD34^+$ and $CD33^+$ bone marrow cells. We used a competitive biotinylated transcription factor oligonucleotide binding NoShift assay (Novagen), which is a colorimetric assay similar to an electrophoretic mobility shift assay, and a ChIP assay. Details are in **Supplementary Methods** and **Supplementary Table 2** online.

Lentiviral transduction of $CD34^+$, K562 and HL-60 cells. Construction of *LEF1* cDNA, dn *LEF1* cDNA and shRNA-containing lentiviral vectors are described in the **Supplementary Methods**. We prepared recombinant lentiviral supernatants as described previously[36]. The virus titers averaged and typically ranged from 1×10^8 to 5×10^8 IU/ml. We transduced $CD34^+$ cells from three healthy donors, HL-60 and K562 cells (1×10^5/well) with lentiviral supernatants with a multiplicity of infection of 1–2, as described previously[36]; re-transduced after 12–24 h; and assessed transduction efficiency after 72 h as the percentage of green fluorescent protein (GFP)- or red fluorescent protein (RFP)-positive cells analyzed by fluorescence-activated cell sorting (FACS). We used virus-free conditioned medium from nontransfected cells as a control.

***In vitro* proliferation and granulocytic differentiation experiments.** We cultured 1×10^5 transduced and sorted RFP^+ or GFP^+ $CD34^+$ cells in X-VIVO 10 medium (Cambrex) supplemented with 20 ng/ml of interleukin-3 (IL-3), 20 ng/ml of interleukin-6 (IL-6), 20 ng/ml of thrombopoietin, 50 ng/ml of stem cell factor and 50 ng/ml of Flt3 ligand, all purchased from R&D Systems. We cultured HL-60 and K562 cells (1×10^5 per well) in supplemented RPMI 1640–10% FCS medium. To assess proliferation, we counted viable cells by trypan blue dye exclusion in a hemocytometer and measured 5-bromo-2-deoxyuridine (BrdU) uptake using BrdU Flow Kit (Pharmingen). We determined the percentage of apoptotic cells using annexin V–FITC conjugate (Pharmingen) and by counting apoptotic cells with morphological evidence of apoptosis (chromatin condensation and fragmented nuclei) on cytospin preparations. For granulocytic differentiation, we cultured 1×10^5 GFP^+-transduced $CD34^+$ cells from two CN-affected patients in supplemented RPMI 1640–1% FCS medium in the presence of G-CSF (10 ng/ml). We characterized granulocytic differentiation by FACS analysis of cells stained with phycoerythrin-conjugated CD15-specific (Caltag) and phycoerythrin-conjugated CD11b-specific (Pharmingen) antibody and by morphological assessment of Wright-Giemsa–stained cytospin slides.

Western blot analysis. We used the following antibodies: mouse monoclonal LEF-1 (REMB1, Calbiochem), rabbit polyclonal LEF-1 antiserum (R.G.), mouse monoclonal β-catenin (BD Transduction Laboratories), rabbit monoclonal β-actin (Santa Cruz Biotechnology) and secondary anti-mouse or anti-rabbit horseradish peroxidase–conjugated antibody (Santa Cruz Biotechnology). We obtained whole cell lysates either through lysis of a defined number of cells in lysis buffer or through direct disruption in Laemmli loading buffer followed by brief sonication. We separated proteins by 10% SDS-PAGE and probed the blots either for 1 h at 24 °C or overnight at 4 °C.

Statistical analysis. We performed statistical analysis using the SPSS V. 9.0 statistical package (SPSS) and a two-sided unpaired Student *t*-test for the analysis of differences in mean values between groups.

GenBank accession numbers. Human *LEF1* (encoding LEF-1), NM_016269; human *CTNNA1* (encoding β-catenin), NM_007614; *CEBPA* (encoding C/EBPα) promoter, S75265.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

K.W., G.C., J.S. and M.St. made initial observations. J.S. designed and performed the main experiments, analyzed the data and wrote the manuscript. M.U. performed western blot analysis and wrote the manuscript. M.Sch., M.E. and K.B. designed shRNA constructs and transduced cells. A.S. and C.B. designed lv constructs that contained full-length LEF-1 or dnLEF-1 and produced viral supernatants. M.G. performed sequence analysis of ELA2 gene. C.Z. provided patients data. U.L. provided laser-assisted microscope. K.W. supervised experimentation, coordinated the project and wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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Erratum: LEF-1 is crucial for neutrophil granulocytopoiesis and its expression is severely reduced in congenital neutropenia

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