Tnpo3 enables EBF1 function in conditions of antagonistic Notch signaling

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Transcription factor EBF1 (early B cell factor 1) acts as a key regulator of B cell specification. The transcriptional network in which EBF1 operates has been extensively studied; however, the regulation of EBF1 function remains poorly defined. By mass spectrometric analysis of proteins associated with endogenous EBF1 in pro-B cells, we identified the nuclear import receptor Transportin-3 (Tnpo3) and found that it interacts with the immunoglobulin-like fold domain of EBF1. We delineated glutamic acid 271 of EBF1 as a critical residue for the association with Tnpo3. EBF1^{E271A} showed normal nuclear localization; however, it had an impaired B cell programming ability in conditions of Notch signaling, as determined by retroviral transduction of *Ebf1*$^{-/-}$ progenitors. By RNA-seq analysis of EBF1^{E271A}-expressing progenitors, we found an up-regulation of T lineage determinants and down-regulation of early B genes, although similar chromatin binding of EBF1^{E271A} and EBF1^{wt} was detected in pro-B cells expressing activated Notch1. B lineage-specific inactivation of *Tnpo3* in mice resulted in a block of early B cell differentiation, accompanied by a down-regulation of B lineage genes and up-regulation of T and NK lineage genes. Taken together, our observations suggest that Tnpo3 ensures B cell programming by EBF1 in nonpermissive conditions.

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The commitment of hematopoietic progenitors to the B cell lineage requires the activity of lineage-specific transcription factors that induce a B lineage-specific gene expression program and/or repress genes associated with alternative cell fates (for reviews, see Nutt and Kee 2007; Boller and Grosschedl 2014; Rothenberg 2014). Multipotent progenitors differentiate in the fetal liver and in the bone marrow via common lymphoid progenitors, pre-pro-B cells, pro-B cells, and pre-B cells to immature B cells that display an antigen-specific receptor on their cell surface. This developmental process includes multilineage priming of enhancers, which provides permissive chromatin states for the action of lineage-specific transcription factors (Heinz et al. 2010; Mercer et al. 2011; Strid et al. 2021). Moreover, a regulatory network—consisting of the transcription factors E2A, Foxo1, EBF1, and Pax5—has been implicated in the specification of the B cell lineage and the activation of B cell genes (Medina et al. 2004; Lin et al. 2010; Mansson et al. 2012). In this network, EBF1 has a lineage-instructive function because it is sufficient to overcome an early differentiation block of progenitors lacking the transcription factor Ikaros (Reynaud et al. 2008). EBF1 acts as a pioneer transcription factor that binds naïve, inaccessible chromatin and mediates a de novo gain of chromatin accessibility by recruiting chromatin remodelers (Maier et al. 2004; Boller et al. 2016; Li et al. 2018; Wang et al. 2020; Strid et al. 2021). In addition, Pax5 and EBF1 are also involved in committing cells to the B cell fate by repressing genes associated with alternative lineage potential (Nutt et al. 1999; Cobaleda et al. 2007; Nechanitzky et al. 2013). In particular, Pax5 represses genes encoding cell surface receptors, such as Notch1 and CSF1R, whereas EBF1 represses genes coding for T lineage-promoting transcription factors, such as TCF1 and GATA3 (Souabni et al. 2002; Revilla et al. 2012; Banerjee et al. 2013; Nechanitzky et al. 2013).

Notch1 is a key determinant for specifying the T lineage that is activated in early thymic progenitors (ETPs) by the interaction with ligands of Delta-like family on stromal cells of the thymus (for reviews, see Maillard et al. 2005; Tanigaki and Honjo 2007; Radtke et al. 2013). This interaction leads to the cleavage of the Notch intracellular

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domain (NICD), which interacts with the transcription factor RBPJ on Notch-responsive genes (Jarriault et al. 1995). NICD activates the genes encoding the T lineage transcription factors TCF1 and GATA3, which activate T cell-specific gene expression in combination with other transcription factors (Hozumi et al. 2008; Germar et al. 2011). Notch signaling in ETPs results in the repression of *Ebf1* and *Pax5* (Zhang et al. 2012), and Notch1 has been implicated in antagonizing the function of EBF1 in transient transfection assays (Smith et al. 2005). Thus, the cross-antagonistic features of EBF1 and Notch signaling may govern the early cell fate decisions of developing lymphocytes.

The transcriptional network in which EBF1 acts to regulate B lineage specification and commitment has been extensively studied; however, the regulation of EBF1 function by protein interactions and modifications remains poorly understood. Previous studies identified interactions of EBF1 with the multi-zinc finger proteins ZNF423 and ZNF521 (Tsai and Reed 1997; Mega et al. 2011; Mesuraca et al. 2015). ZNF423, also termed Roaz, interacts with the helix–loop–helix (HLH) domain of EBF1, thus preventing EBF1 dimerization and DNA binding. Transcription factor MEF2c has also been identified as an EBF1-interacting protein, and 72% of genomic MEF2c-binding sites in pre-B cells were found to overlap with EBF1-binding sites, suggesting a functional collaboration between these transcription factors (Kong et al. 2016). In addition, the interaction of EBF1 with the CCR4/NOT complex has been suggested to diversify the function of EBF1 in a context-dependent manner and to facilitate the coordination of transcriptional and post-transcriptional gene regulation (Yang et al. 2016). Finally, Brg1 (a component of the SWI/SNF chromatin remodeling complex) and the FET family proteins FUS, EWSR1, and TAF15 were found to interact with the C-terminal domain of EBF1 and mediate chromatin accessibility at EBF1-occupied sites (Wang et al. 2020).

Here, we report the interaction of EBF1 with Tnpo3 (Transportin-3 or Transportin-SR2), a nuclear import receptor that transports SR proteins into the nucleus and thus indirectly controls pre-mRNA splicing (Kataoka et al. 1999; Lai et al. 2000, 2001). Conditional inactivation of *Tnpo3* in early T cells of mice was previously shown to result in a partial block of early T cell differentiation, accompanied by reduced TCR signaling in $CD8^+$ T cells (Iwanami et al. 2016). In this study, we found that Tnpo3 interacts with EBF1 as a nuclear partner and identified glutamic acid 271 in the EBF1 immunoglobulin–plexin–transcription factor (IPT) domain as a critical residue for the interaction. The mutation of this residue did not affect the nuclear localization of EBF1 but attenuated EBF1 target gene expression and resulted in a partial block of differentiation of pre-pro-B cells to early pro-B cells in conditions of T lineage-promoting Notch signaling. Moreover, a B cell-specific deletion of *Tnpo3* in mice revealed a block of early B cell differentiation in the bone marrow, which was accompanied by a diminished expression of B cell-specific EBF1 target genes and increased expression of T cell lineage-associated genes. Thus, our findings suggest that the interaction of Tnpo3 with EBF1 enables efficient B cell programming in nonpermissive conditions.

Results

EBF1 associates with Tnpo3 in pro-B cells

Stable isotope labeling with amino acids in cell culture (SILAC) and mass spectrometry of proteins that interact with endogenous EBF1 in A-MuLV transformed 38B9 pro-B cells identified most subunits of the CCR4–NOT complex (Yang et al. 2016). In a reanalysis of these mass spectrometry data sets, which included six forward and reverse SILAC-labeling experiments, we also detected an enrichment of the nuclear import receptor Transportin-3 (Tnpo3) at levels comparable with those observed for the components of the CCR4–NOT complex (Supplemental Fig. S1A,B). The association of Tnpo3 with EBF1 was also detected in samples treated with benzonase nuclease, indicating that this protein interaction was independent of DNA or RNA (Supplemental Fig. S1A,B). We confirmed the interaction of Tnpo3 with EBF1 by coimmunoprecipitation (co-IP) in whole-cell extracts of 38B9 pro-B cells (Fig. 1A). To determine whether the interaction occurs in the nucleus, we also used nuclear extracts of pro-B cells and observed a coimmunoprecipitation of Tnpo3 with EBF1 (Fig. 1B). Taken together, these experiments suggested that EBF1 interacts with Tnpo3.

EBF1 interacts with Tnpo3 via glutamic acid 271 in the IPT domain of EBF1

To confirm the specificity of this interaction, we aimed at identifying the protein domain and amino acids within EBF1 that are critical for the contact with Tnpo3. EBF1 harbors a transcription factor immunoglobulin (TIG/IPT) domain similar to those of NFAT and NFκB, which have been suggested to mediate protein–protein interaction (Cramer and Müller 1999; Treiber et al. 2010a). In addition, EBF1 contains a C-terminal domain (CTD) that has been shown to be important for the ability of EBF1 to bind inaccessible, naïve chromatin and induce target gene expression (Boller et al. 2016; Wang et al. 2020). Therefore, we generated EBF1 mutants lacking either of these domains (Fig. 1C). We generated and transfected plasmids expressing these mutants into HEK293 cells and examined the EBF1:Tnpo3 interaction by co-IPs. The deletion of the CTD had no effect on the association of EBF1 with Tnpo3, whereas the deletion of the IPT domain abrogated this interaction (Fig. 1D). To investigate whether specific residues in the IPT domain may facilitate the interaction with Tnpo3, we analyzed X-ray crystallography data of DNA-bound EBF1ΔCTD (Treiber et al. 2010a). This analysis revealed the presence of a glutamic acid residue at position 271 that notably protruded from the protein surface of the IPT domain (Fig. 1E). Alignment of the protein sequences of EBF1 from *Mus musculus* and its orthologs Collier from *D. melanogaster* and Unc-3 from *C. elegans* indicated higher conservation of amino acids between positions 269 and 273 than in the flanking

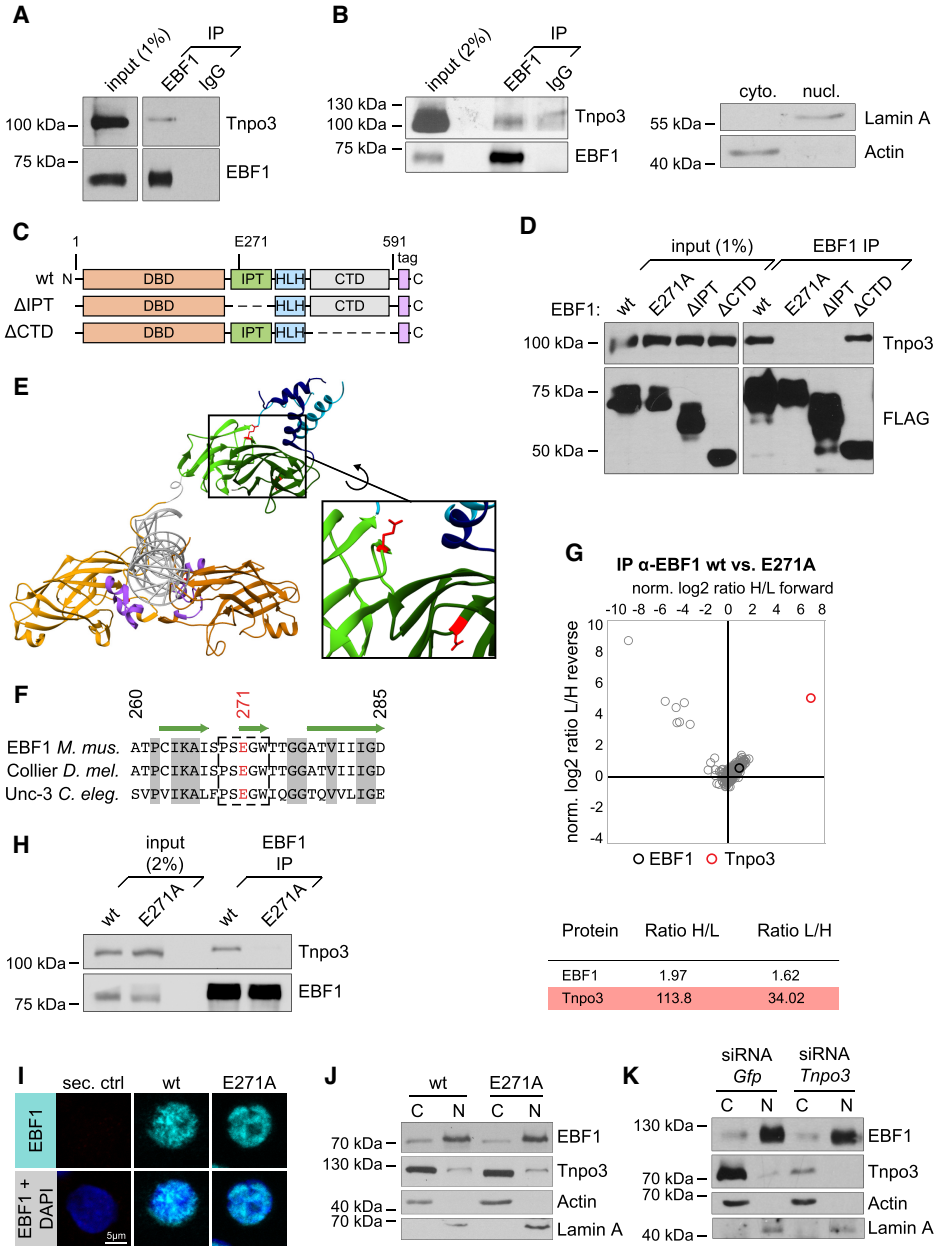

Figure 1. Tnpo3 interacts with EBF1 via glutamic acid 271 in the IPT domain. (*A*,*B*) Coimmunoprecipitation of Tnpo3 with endogenous EBF1 from whole-cell extracts (*A*) or nuclear extracts (*B*) of A-MuLV transformed pro-B cells. (*C*) Domain structure of EBF1 and mutant derivatives. (DBD) DNA-binding domain, (IPT) immunoglobulin–plexin–transcription factor domain, (HLH) helix–loop–helix domain, (CTD) C-terminal domain. The C-terminal Strep-FLAG tag is indicated in purple. (*D*) Coimmunoprecipitation of Tnpo3 with Strep-FLAG-tagged EBF1 proteins from lysates of HEK293 cells transfected with EBF1 expression plasmids. (*E*) Crystal structure of the murine DNA-bound EBF1 homodimer lacking the unstructured CTD (Treiber et al. 2010a). Part of the IPT domain (green) with glutamic acid E271 (red) is highlighted. The monomers are indicated in light or dark colors, and the zinc-binding motif is shown in purple. DNA is depicted in gray. (*F*) Protein sequence alignment of amino acids 260 and 285 in the EBF1 IPT domain from *Mus musculus* with the corresponding sequences of the orthologs Collier from *D. melanogaster* and Unc-3 from *C. elegans*. E271 (red) resides in a short region of sequence identity (dashed square). Additional conserved amino acids are highlighted in gray, and β sheets are indicated by green arrows. (*G*) Scatter plot depicting $\log_2$ values of normalized SILAC ratios of forward and reverse labeling experiments, comparing proteins coimmunoprecipitated with EBF1^{wt} versus EBF1^{E271A} from pro-B cell lysates. Normalized SILAC ratios of the relative enrichment of EBF1 and Tnpo3 are indicated *below*. (*H*) Coimmunoprecipitation of Tnpo3 with Strep-FLAG-tagged EBF1^{wt} or EBF1^{E271A} from lysates of primary pro-B cells in which the endogenous *Ebf1* gene has been deleted. (*I*) Immunofluorescence staining of EBF1^{wt} or EBF1^{E271A} (cyan) and DAPI (blue) in primary pro-B cells lacking the endogenous *Ebf1* gene. EBF1^{wt}-expressing cells that were stained only with a secondary antibody served as a negative control. (*J*) Immunoblot analysis of subcellular fractions of A-MuLV transformed EBF1^{wt} or EBF1^{E271A} pro-B cells lacking the endogenous *Ebf1* gene. (C) Cytoplasmic fraction, (N) nuclear fraction. (*K*) Immunoblot analysis of subcellular fractions obtained from 38B9 pro-B cells that were transduced with *Tnpo3* siRNA or *Gfp* siRNA control.

protein sequences (Fig. 1F), suggesting that this region may be critical for the function of EBF1. Indeed, replacement of glutamic acid 271 with alanine abrogated the interaction with Tnpo3 in co-IP experiments with lysates of HEK293 cells that were transfected with an EBF1^{E271A} expression plasmid (Fig. 1D).

To assess the specificity of the EBF1:Tnpo3 interaction in a more physiological context, we used a gene replacement approach to substitute endogenous EBF1 with EBF1^{E271A} in pro-B cells. To this end, we transduced A-MuLV transformed pro-B cells, derived from *Ebf1*$^{fl/fl}$ *RERT*^{Cre} mice (György et al. 2012), with retroviruses expressing EBF1^{wt} or EBF1^{E271A}, selected for retroviral expression, and treated the cells with 4-hydroxy-tamoxifen to delete the endogenous *Ebf1* alleles. We labeled EBF1^{wt}- and EBF1^{E271A}-expressing pro-B cells with heavy or light amino acids and compared immunoprecipitated EBF1-associated proteins in a forward and reverse labeling mode by SILAC-based mass spectrometry (Fig. 1G). This analysis confirmed the loss of Tnpo3 association with EBF1^{E271A} relative to EBF1^{wt}. Notably, Tnpo3 was the only identified protein that showed a differential interaction with EBF1^{wt} and EBF1^{E271A}, suggesting that Tnpo3 does not act as an adapter protein for additional interaction partners. Some proteins that were depleted in EBF1^{wt} samples were identified as contaminating keratins (data not shown). We also confirmed the impaired interaction of EBF1^{E271A} with Tnpo3 by co-IP in primary *Ebf1*$^{-/-}$ pro-B cells complemented with EBF1^{wt}- or EBF1^{E271A}-expressing retroviruses (Fig. 1H). These data indicate that glutamic acid 271 within the EBF1 IPT domain is a critical residue for the interaction with Tnpo3, and its evolutionary conservation suggests a functional relevance.

Loss of the Tnpo3 interaction does not alter the nuclear localization of EBF1

Tnpo3 is a nuclear import receptor that mediates the nuclear localization of serine/arginine-rich (SR) proteins. Therefore, we examined whether Tnpo3 facilitates the nuclear import of EBF1. To this end, we transduced primary fetal liver-derived *Ebf1*$^{-/-}$ c-kit^{+} hematopoietic stem and progenitor cells (HSPCs) with bicistronic retroviruses that express EBF1^{wt} or EBF1^{E271A} along with GFP. We sorted transduced and GFP-expressing cells by flow cytometry and analyzed the nuclear localization of EBF1 by immunofluorescence (Fig. 1I). EBF1^{wt} and EBF1^{E271A} were both detected in the DAPI-dense nuclear region, and the quantification of nuclear EBF1 fluorescent signals showed equal nuclear accumulation of EBF1^{wt} and EBF1^{E271A} (Fig. 1I; Supplemental Fig. S1C). We also analyzed the nuclear accumulation of EBF1^{wt} or EBF1^{E271A} in pro-B cells by subcellular fractionation and immunoblot analysis. For this experiment, we transduced EBF1^{wt}- or EBF1^{E271A}-expressing retroviruses into A-MuLV transformed *Ebf1*$^{fl/fl}$*RERT*^{Cre} pro-B cells in which the endogenous *Ebf1* alleles were deleted by tamoxifen treatment after transduction. Similar amounts of EBF1^{wt} and EBF1^{E271A} proteins were predominantly detected in the nuclear fractions, whereas Tnpo3 protein was mainly detected in the cytoplasmic fractions (Fig. 1J). We also examined the effects of a siRNA-mediated knockdown of *Tnpo3* on the subcellular distribution of endogenous EBF1 in 38B9 pro-B cells. Endogenous EBF1 accumulated to a similar extent in nuclear fractions of pro-B cells transduced with *Tnpo3* siRNA-expressing retrovirus as compared with a *Gfp* siRNA transduced control (Fig. 1K). Taken together, these results indicate that the nuclear localization of EBF1 does not depend on Tnpo3, raising the possibility that Tnpo3 has a function in association with EBF1 independent of its role as a nuclear import receptor.

Loss of EBF1:Tnpo3 interaction impairs B cell programming in nonpermissive conditions

To investigate whether Tnpo3 regulates the function of EBF1 in B cell programming, we transduced fetal liver-derived and sorted *Ebf1*$^{-/-}$ c-kit^{+} HSPCs with GFP-bicistronic retroviruses expressing EBF1^{wt} or EBF1^{E271A}. We sorted GFP^{+} progenitors and cocultured them with OP9 feeder cells in B cell-promoting conditions for 7 d. Immunoblot analysis showed comparable expression levels of EBF1^{wt} and EBF1^{E271A} and the absence of endogenous EBF1 in vector transduced cells (Supplemental Fig. S1D). Moreover, a flow cytometric analysis of intracellular EBF1 indicated a homogeneous expression of EBF1^{wt} and EBF1^{E271A} at similar levels in individual cells (Supplemental Fig. S1E). Flow cytometric analysis of B lineage surface maker expression revealed that 92% of EBF1^{E271A}-expressing cells and 91% of EBF1^{wt}-expressing cells displayed CD19 on their cell surface (Fig. 2A, top panels). Further analysis of markers for early and late pro-B cell stages showed similar frequencies of CD19^{+}BP1^{-} early pro-B cells (Fr.B) and CD19^{+}BP1^{+} late pro-B cells (Fr.C) in EBF1^{E271A}-expressing cells relative to EBF1^{wt}-expressing cells (Supplemental Fig. S2A–C). These data indicate that the function of EBF1^{E271A} in B cell programming was similar to that of EBF1^{wt} in OP9 cocultures.

Although Notch overexpression has been shown to antagonize the function of EBF1 (Smith et al. 2005), EBF1^{wt} can override the effects of Notch signaling in B cell programming (Boller et al. 2016). To assess the function of EBF1^{E271A} in the nonpermissive conditions of Notch signaling, we cocultured EBF1^{wt}- and EBF1^{E271A}-expressing progenitors with T cell-promoting OP9-DL1 feeder cells that display the Notch ligand Delta-like protein 1 (DL1) on their cell surface (Taghon et al. 2005). After 7 d of coculture, EBF1^{E271A}-expressing cells showed a reduced frequency of CD19^{+} pro-B cells relative to EBF1^{wt}-expressing cells and a reciprocal increase in the frequency of CD19^{-} cells displaying the early T cell marker CD25 on their surface (Fig. 2A, middle panels). Quantification of multiple experiments revealed an ~50% decrease in the frequencies of CD19^{+} pro-B cells and an approximately fourfold increase in the frequencies of CD25^{+}CD19^{-} cells in EBF1^{E271A} versus EBF1^{wt} cocultures on OP9-DL1 feeders (Fig. 2B; Supplemental Fig. S2D). To assess whether the increase in the frequencies of CD25^{+}CD19^{-} cells in EBF1^{E271A} cocultures could be due to the expansion of

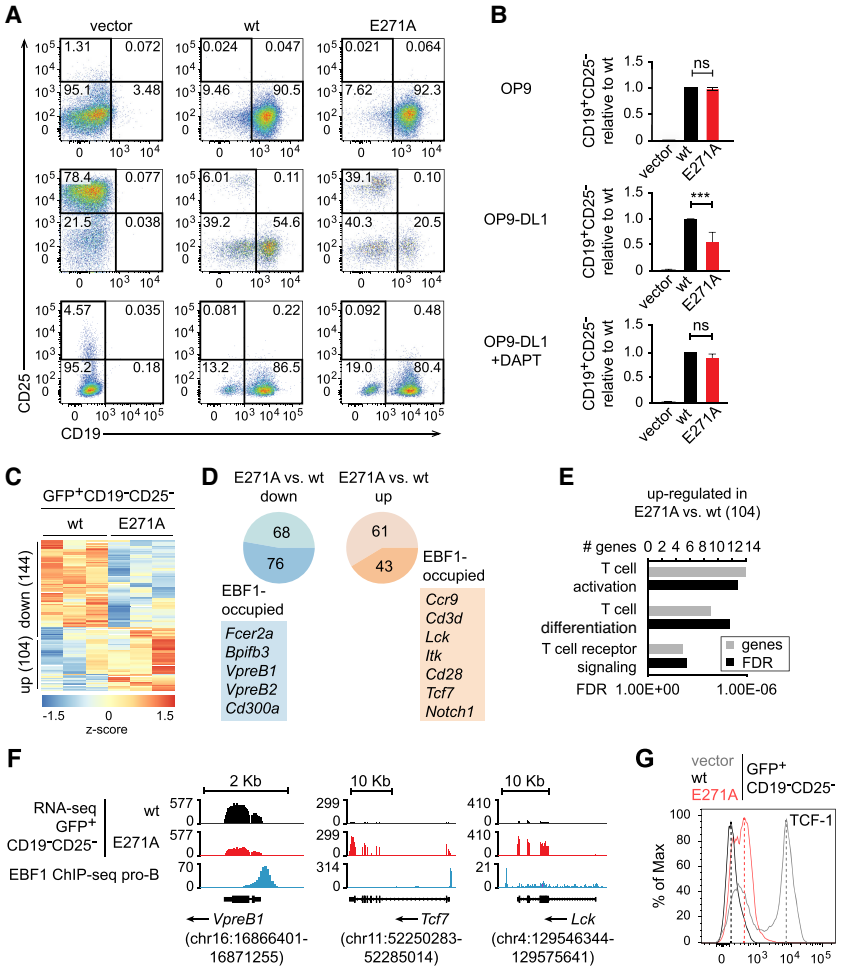

Figure 2. Differentiation of EBF1^{E271A} transduced HSPCs in OP9 or OP9-DL1 cocultures. (*A*) Flow cytometry analysis of CD19 and CD25 surface expression on *Ebf1*$^{-/-}$ ckit$^+$ HSPCs transduced with bicistronic retroviruses expressing EBF1^{wt} or EBF1^{E271A} along with GFP. GFP$^+$ cells were cocultured for 7 d with OP9 (*top*) or OP9-DL1 feeder cells (*middle*), or with OP9-DL1 feeders in the presence of the Notch inhibitor DAPT (*bottom*). Numbers represent the percentages of cells within the gates. (*B*) Frequencies of CD19$^+$CD25$^-$ cells obtained from independent cocultures with OP9 (*top*), OP9-DL1 (*middle*), or OP9-DL1 feeders with DAPT (*bottom*). Bars represent means ± SD of frequencies relative to EBF1^{wt} (OP9, $n = 8$; OP9-DL1, $n = 12$; OP9-DL1 + DAPT, $n = 4$). Unpaired *t*-test was used for hypothesis testing. (***) $P < 0.001$, (ns) not significant ($P > 0.05$). (*C*) Heat map of the 248 genes significantly deregulated ($q < 0.1$) in EBF1^{E271A}-expressing versus EBF1^{wt}-expressing GFP$^+$CD19$^-$CD25$^-$ cells cocultured with OP9-DL1 feeders. Clusters with down-regulated and up-regulated genes are indicated. Expression values are scaled to the row *z*-score, and three biological replicates were analyzed. (*D*) Relative numbers of down-regulated and up-regulated genes that are occupied by EBF1, as determined by ChIP-seq analysis (Treiber et al. 2010b; Boller et al. 2016; Li et al. 2018). Representative genes are indicated. (See Supplemental Table S1 for a full list of genes.) (*E*) Gene ontology (GO) term analysis of the 104 up-regulated genes in EBF1^{E271A}-expressing versus EBF1^{wt}-expressing GFP$^+$CD19$^-$CD25$^-$ cells. (*F*) Genome browser tracks showing the expression of *VpreB1* (*left*), *Tcf7* (*middle*), and *Lck* (*right*) in GFP$^+$CD19$^-$CD25$^-$ cells and EBF1 occupancy in wild-type pro-B cells, as determined by ChIP-seq (Boller et al. 2016). (*G*) Intracellular flow cytometric analysis of TCF-1 in vector, EBF1^{wt}, or EBF1^{E271A} transduced GFP$^+$CD19$^-$CD25$^-$ cells cocultured with OP9-DL1 feeders for 7 d. The dashed lines indicate the fluorescence intensity at the maximal peak height.

contaminating T or NK lineage precursors in conditions of Notch signaling, we examined the c-kit$^+$ progenitor population used for retroviral transduction for the presence of cells expressing CD3 and/or NK1.1. Flow cytometric analysis indicated that <1% of the c-kit$^+$ progenitors expressed one or both of these surface markers (Supplemental Fig. S2E). However, we cannot rule out that a small fraction of the T cells may come from contaminating T lineage precursors.

To provide further evidence for a role of Notch signaling in antagonizing the B lineage programming by EBF1^{E271A}, we examined whether the differentiation defect of EBF1^{E271A}-expressing progenitor cells in coculture with OP9-DL1 feeders could be rescued by the addition of the γ-secretase inhibitor DAPT. This small molecule inhibits the proteolytic cleavage of the Notch1 receptor and release of the NICD into the nucleus (Geling et al. 2002). The addition of DAPT allowed for a similar generation of CD19$^+$ pro-B cells in EBF1^{E271A} and EBF1^{wt} OP9-DL1 cocultures, indicating that the partial differentiation block in EBF1^{E271A}-expressing cells was dependent on Notch signaling (Fig. 2A, bottom panels). Thus, EBF1^{E271A} has a reduced potential to generate early pro-B cells from progenitors in conditions of antagonistic Notch signaling, suggesting that Tnpo3 supports EBF1 to enforce B cell programming in nonpermissive conditions.

EBF1^{E271A}-expressing progenitor cells show a T cell lineage-biased transcriptome

To gain insight into the molecular basis of the impaired B cell programming of EBF1^{E271A} cells in conditions of Notch signaling, we determined the transcriptome of EBF1^{wt} and EBF1^{E271A} transduced progenitor cells by RNA-seq analysis. For this analysis, we sorted GFP$^+$CD19$^-$CD25$^-$ cells to analyze the gene expression profiles in cells preceding the differentiation block. In comparison with EBF1^{wt}-expressing cells, EBF1^{E271A} mutant cells showed the down-regulation and up-regulation of 144 and 104 genes, respectively (Fig. 2C). By overlapping the data set of EBF1^{E271A}-deregulated genes with data sets of EBF1 occupancy in pro-B cells (Treiber et al. 2010b; Boller et al.

2016; Li et al. 2018), we identified 76 out of 144 down-regulated and 43 out of 104 up-regulated genes (Fig. 2D; Supplemental Table S1). Among the down-regulated and EBF1-occupied genes, we identified *VpreB1* and *VpreB2*, encoding surrogate light chains of the pre-B cell receptor; *Tnfrsf13b*, encoding the TACI receptor of the BAFF ligand; and *Fcer2a* (CD23). Moreover, several genes in the group of down-regulated genes, including *Clic6*, *Eps8l2*, *Gal*, and *Bpifb3*, have been previously found to be among the earliest EBF1-activated targets in a time-resolved analysis of induced EBF1 expression (Li et al. 2018). As expected, the typical EBF1-activated genes, such as *Cd19*, *Pax5*, and *Cd79a*, were not found to be expressed in the EBF1 transduced progenitor cells, as these genes were found to be activated by EBF1 at later time points (Li et al. 2018). In the group of up-regulated genes, we found *Notch1*, *Tcf7*, *Cd3d*, *Cd28*, *Lck*, *Lat*, *Ccr7*, *Ccr9*, and other genes associated with T cell differentiation, activation, and T cell receptor signaling (Fig. 2D–F). *Tcf7* encodes the T lineage-determining and WNT-responsive transcription factor TCF1 (Yu et al. 2009; Weber et al. 2011; Johnson et al. 2018), which is repressed by EBF1 in pro-B cells (Nechanitzky et al. 2013; Li et al. 2018). To examine whether the up-regulation of *Tcf7* reflects a derepression in all or a subset of cells, we performed intracellular FACS analysis for TCF1. In the majority of EBF1^{E271A} transduced cells, we observed an augmented TCF1 expression relative to EBF1^{wt} transduced cells (Fig. 2G). However, the TCF1 protein level in EBF1^{E271A} transduced cells was lower than the level detected in vector transduced cells, suggesting that the EBF1^{E271A}-expressing cells may have a mixed lineage potential rather than a full lineage-converted phenotype. The tyrosine protein kinase Lck is an important component of the TCR/CD3 signaling pathway (Barber et al. 1989), and the genes encoding the chemokine receptors CCR7 and CCR9 mediate the homing of hematopoietic progenitors to the thymus (Zlotoff et al. 2010; Calderón and Boehm 2011). Thus, the molecular signature of EBF1^{E271A}-expressing CD19$^-$CD25$^-$ cells confirmed the shift in the lineage potential of these cells relative to EBF1^{wt}-expressing CD19$^-$CD25$^-$ cells.

EBF1^{E271A}-dependent transcriptome changes do not reflect altered EBF1 gene occupancy

To further assess the effects of EBF1^{E271A} expression on B cell programming in the absence or presence of Notch1 signaling, we also analyzed the transcriptome of EBF1-expressing cells at an early time point preceding the appearance of CD19$^+$ early pro-B cells. To this end, we sorted EBF1^{wt} and EBF1^{E271A} transduced progenitors for GFP expression 16 h after transduction and cocultured them with OP9 or OP9-DL1 feeders for 3 d. RNA-seq analysis of EBF1^{E271A}- and EBF1^{wt}-expressing cells, cocultured on OP9-DL1 feeders, identified 44 differentially expressed genes ($q < 0.1$), of which 29 were down-regulated and 15 were up-regulated in EBF1^{E271A} versus EBF1^{wt} cells (Fig. 3A). Interrogation of the RNA-seq data with previous genome-wide EBF1 ChIP data (Treiber et al. 2010b; Boller et al. 2016; Li et al. 2018) revealed that 23 down-regulated genes and five up-regulated genes show EBF1 occupancy in pro-B cells and in *Ebf1*$^{-/-}$ progenitors upon induction of EBF1 expression (Fig. 3B; Supplemental Table S2). Among the down-regulated EBF1 target genes, we identified the B lineage genes *Pou2af1*, *VpreB1*, *VpreB3*, *Fcer2a*, and *Igll1*, which play crucial roles in B cell development and function (Kehry and Yamashita 1989; Kitamura et al. 1992; Nielsen et al. 1996; Schubart et al. 1996; Mundt et al. 2001). We validated the down-regulation of these and other EBF1-occupied genes in multiple EBF1^{E271A} short-term cultures on OP9-DL1 feeders by qRT-PCR analysis (Fig. 3C). In this analysis, we also included EBF1-occupied genes, such as *Asb2*, *Otub2*, *Pax5*, and *Cd79b*, which were expressed at similar levels in EBF1^{E271A} and EBF1^{wt} cultures. We also analyzed the expression of these genes in EBF1^{E271A} and EBF1^{wt} transduced progenitors that were cocultured with OP9 feeders. In EBF1^{E271A}:OP9 cocultures, we detected a modest down-regulation of some genes (*Matn1*, *Fcer2a*, and *Lef1*) that were strongly down-regulated in EBF1^{E271A}:OP9-DL1 cocultures (Supplemental Fig. S2F). This observation suggests that EBF1^{E271A} has a compromised potency to activate specific B lineage genes that is further attenuated by Notch signaling.

To address the question of whether the deregulation of EBF1 target genes in EBF1^{E271A}-expressing cells was caused by altered EBF1^{E271A} occupancy, we performed a quantitative EBF1 ChIP-seq analysis. Toward this end, we used EBF1^{E271A}- and EBF1^{wt}-expressing primary pro-B cells in which the expression of the NICD could be induced by doxycycline (Dox) after 6 h. To obtain sufficient cells for ChIP-seq analysis, we had to use an inducible system because a prolonged coculture of EBF1 transduced cells on OP9-DL1 feeders resulted in a loss of EBF1 expression and cell death (data not shown). Immunoblot analysis confirmed comparable EBF1^{wt} and EBF1^{E271A} expression in Dox-treated pro-B cells (Supplemental Fig. S2G). Moreover, a qRT-PCR analysis revealed a strong induction of the Notch target gene *Dtx1* in both Dox-treated EBF1^{wt}- and EBF1^{E271A}-expressing pro-B cells (Supplemental Fig. S2H). The ChIP-seq analysis identified 4116 peaks, of which 3792 (92%) overlapped with previously identified EBF1-bound sites in primary pro-B cells (Supplemental Fig. S2I; Boller et al. 2016). Out of the 4116 peaks, 3919 showed similar intensities in Dox-treated EBF1^{wt}- and EBF1^{E271A}-expressing pro-B cells (Fig. 3D). We also identified small sets of 169 sites with reduced and 28 sites with enhanced peak intensities in EBF1^{E271A}-expressing cells relative to EBF1^{wt}-expressing cells (Fig. 3D; Supplemental Table S3). Thus, EBF1^{E271A} and EBF1^{wt} displayed similar binding at the vast majority of sites in conditions of Notch signaling. We also overlapped these EBF1 ChIP-seq data sets with the RNA-seq data sets of the EBF1^{E271A}- and EBF1^{wt}-expressing short-term OP9-DL1 cocultures. In this analysis, we did not detect a significant overlap between altered gene expression and occupancy, as exemplified by the down-regulated *Pouf2af1* and up-regulated *Prg2* genes (Fig. 3E; data not shown). Conversely, most genes with an altered EBF1^{E271A} occupancy, such as *Exoc6*, were not deregulated (Fig. 3E; data not shown). Thus, the altered target gene expression in EBF1^{E271A}-expressing cells cannot be simply accounted for by changes in EBF1 occupancy.

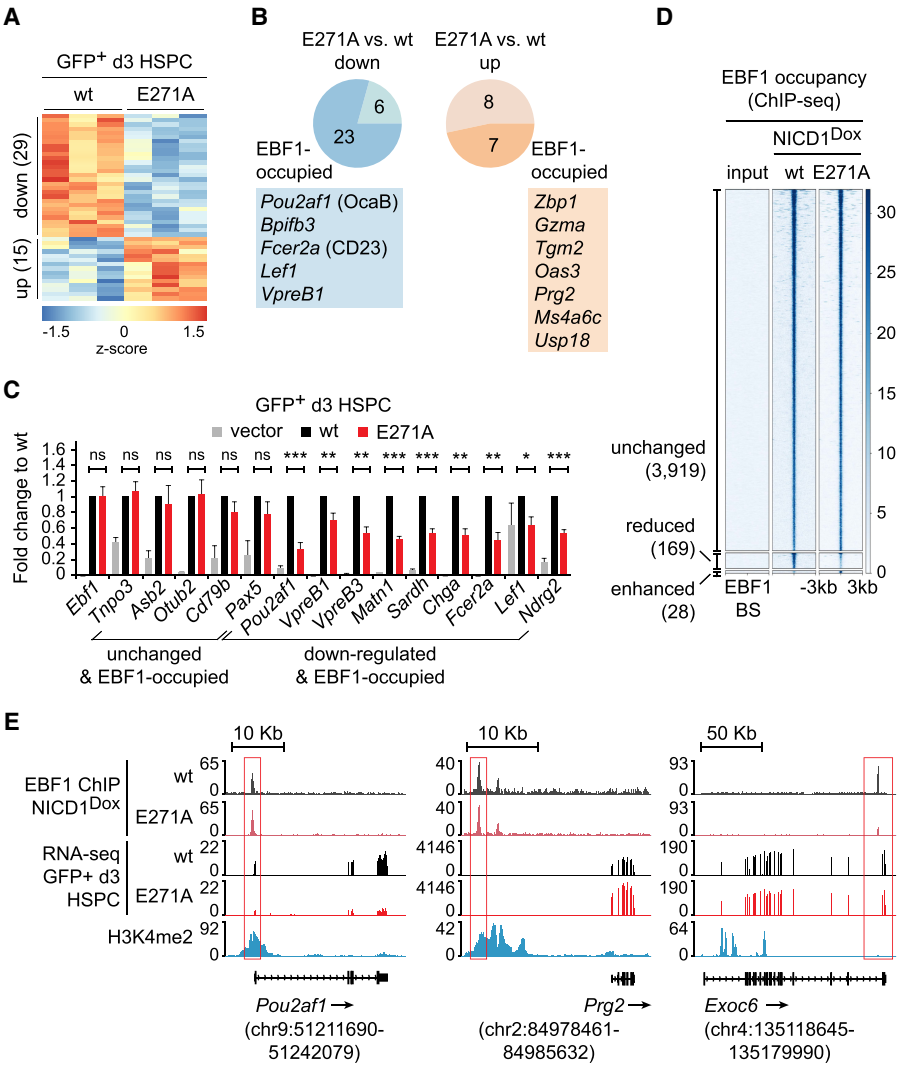

Figure 3. Deregulation of genes in EBF1^{E271A}-expressing cells despite normal chromatin occupancy. (*A*) Heat map of the 44 genes significantly deregulated ($q < 0.1$) in short-term (d3) cultures of EBF1^{E271A} and EBF1^{wt} transduced *Ebf1*$^{-/-}$ ckit^{+} progenitors in coculture with OP9-DL1 feeders. Clusters with down-regulated and up-regulated genes are indicated. Expression values are scaled to the row *z*-score, and three biological replicates were analyzed. (*B*) Relative numbers of down-regulated and up-regulated genes that are occupied by EBF1 as determined by ChIP-seq analysis (Treiber et al. 2010b; Boller et al. 2016; Li et al. 2018). Representative genes are indicated. (See Supplemental Table S2 for a full list of genes.) (*C*) Quantitative RT-PCR analysis to determine the expression of EBF1-occupied genes in EBF1^{E271A} versus EBF1^{wt} short-term cocultures as described in *A*. Expression values were normalized to *Actb* expression and EBF1^{wt}. Bars represent means ± SD of three biological replicates. Unpaired *t*-test was used for hypothesis testing. (***) $P < 0.001$, (**) $P < 0.01$, (*) $P < 0.05$, (ns) not significant ($P > 0.05$). (*D*) Quantitative ChIP-seq analysis to assess binding of EBF1^{wt} and EBF1^{E271A} in primary pro-B cells in which the endogenous *Ebf1* gene has been deleted and NICD1 expression was induced by doxycycline (Dox) treatment for 6 h. Chromatin from human Bjab cells was spiked in for normalization. A region around ±3 kb of EBF1-bound sites (BSs) is shown. The heat map depicts EBF1 peaks that were unchanged, reduced, or enhanced more than twofold in EBF1^{E271A}-expressing relative to EBF1^{wt}-expressing cells. Low-abundance peaks (total read count <100) were excluded from the analysis. (See Supplemental Table S3 for a list of genes with differentially occupied EBF1 peaks.) (*E*) Genome browser tracks showing the EBF1 occupancy of *Pou2af1* (*left*), *Prg2* (*middle*), and *Exoc6* (*right*) in NICD1-expressing pro-B cells and their gene expression in EBF1^{wt}- or EBF1^{E271A}-expressing progenitor short-term cultures on OP9-DL1 feeders. H3K4me2 ChIP-seq signals (blue) of wild-type pro-B cells (Li et al. 2018) mark promoter regions.

B cell-specific deletion of Tnpo3 *results in a differentiation block and in impaired B lineage identity*

To address the role of Tnpo3 in early B lymphopoiesis in vivo, we crossed *Tnpo3*$^{fl/fl}$ and *Tnpo3*$^{+/+}$ mice with *mb1*^{Cre} mice to induce a conditional knockout of *Tnpo3* in early stage B cells. Flow cytometry analysis of the bone marrow of these mice revealed a substantial loss of B220^{hi}CD43^{-} immature/recirculating B cells, B220^{lo}CD43^{-} pre-B cells, B220^{+}CD43^{+}HSA^{+}BP1^{-} early pro-B cells, and B220^{+}CD43^{+}HSA^{+}BP1^{+} late pro-B cells in *Tnpo3*$^{fl/fl}$*mb1*^{Cre} mice (Fig. 4A,B; Supplemental Fig.

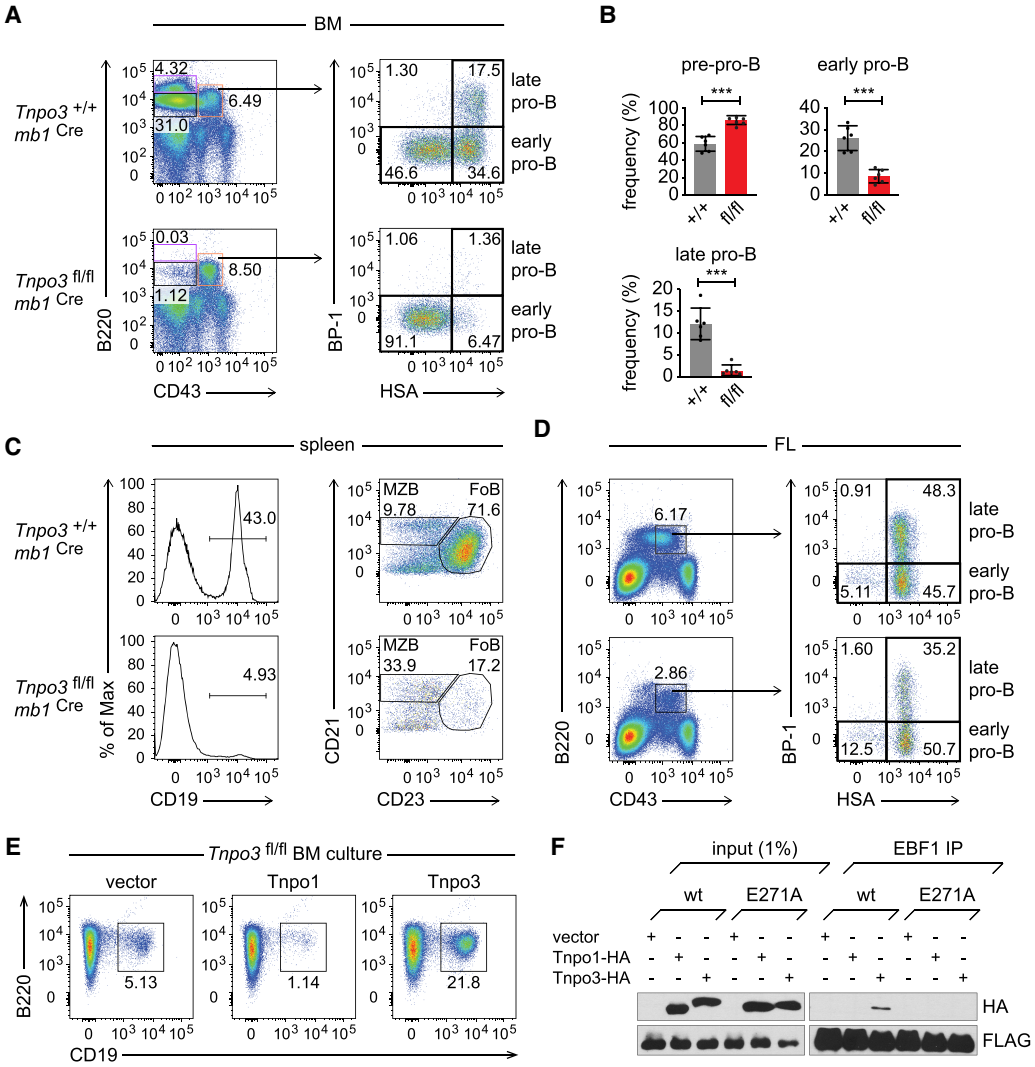

Figure 4. *Tnpo3*$^{fl/fl}$*mb1*^{Cre} mice show impaired B cell differentiation. (*A*) Flow cytometric analysis of bone marrow cells from *Tnpo3*$^{+/+}$*mb1*^{Cre} or *Tnpo3*$^{fl/fl}$*mb1*^{Cre} mice to identify B220^{hi}CD43^{-} recirculating B cells, B220^{lo}CD43^{-} pre-B cells, B220^{+}CD43^{+}HSA^{-}BP1^{-} pre-pro-B cells, B220^{+}CD43^{+}HSA^{+}BP1^{-} early pro-B cells, and B220^{+}CD43^{+}HSA^{+}BP1^{+} late pro-B cells. Numbers represent the percentages of cells within the gates. (*B*) Frequencies of pre-pro-B cells, early pro-B cells, and late pro-B cells in the bone marrow of *Tnpo3*$^{+/+}$*mb1*^{Cre} or *Tnpo3*$^{fl/fl}$*mb1*^{Cre} mice. Dots represent individual mice. Bars represent means ± SD of all animals per group. Unpaired *t*-test was used for hypothesis testing. (***) $P < 0.001$. (*C*) Flow cytometric analysis of CD19^{+} cells (*left* panel) as well as CD21^{hi}CD23^{lo} marginal zone B cells and CD21^{lo}CD23^{hi} follicular B cells (*right* panel) in the spleen of *Tnpo3*$^{+/+}$*mb1*^{Cre} or *Tnpo3*$^{fl/fl}$*mb1*^{Cre} mice. A representative analysis of two biological replicates is shown. (*D*) Flow cytometric analysis of HSA^{-}BP1^{-} pre-pro-B, HSA^{+}BP1^{-} early pro-B, and HSA^{+}BP1^{+} late pro-B cells gated from B220^{+}CD43^{+} cells from the fetal liver of *Tnpo3*$^{+/+}$*mb1*^{Cre} or *Tnpo3*$^{fl/fl}$*mb1*^{Cre} embryos at stage E16.5. $n = 2$. (*E*) Flow cytometric analysis of B220 and CD19 surface expression on *Tnpo3*$^{fl/fl}$*mb1*^{Cre} bone marrow pro-B cells cocultured on OP9 feeders and transduced with retroviruses expressing Tnpo1 or Tnpo3. Analysis was performed 7 d after transduction. One representative experiment of two biological replicates is shown. (*F*) Coimmunoprecipitation of HA-tagged Tnpo1 or Tnpo3 with Strep-FLAG-tagged EBF1 proteins from lysates of HEK293 cells cotransfected with Tnpo1 or Tnpo3 and EBF1 expression plasmids.

S2A). In the mutant mice, we also observed an accumulation of B220^{+}CD43^{+}HSA^{-} pre-pro-B cells. This differentiation block from the pre-pro-B to the early pro-B cell stage was more severe than that observed in the OP9-DL1 coculture experiments.

To determine the phenotype of *Tnpo3* deficiency in peripheral B cell subsets, we also examined splenic B cell populations by flow cytometry and found a marked decrease in the frequencies of CD19^{+} B cells (Fig. 4C). Further flow cytometric analysis indicated a preferential loss of CD21^{lo}CD23^{hi} follicular B cells relative to CD21^{hi}CD23^{lo} marginal zone B cells. We also examined the effects of *Tnpo3* deletion on B lymphopoiesis in the fetal liver. We observed only a modest decrease in the frequencies of B220^{+}CD43^{+}HSA^{+} pro-B cells and a modest increase in the frequencies of B220^{+}CD43^{+}HSA^{-} pre-pro-B cells (Fig. 4D), suggesting a marked difference in the requirement of *Tnpo3* for fetal and adult B lymphopoiesis.

To examine whether the differentiation block in bone marrow B lymphopoiesis of *Tnpo3*$^{fl/fl}$*mb1*^{Cre} mice could be rescued by the re-expression of Tnpo3, we transduced mutant pro-B cell cultures with a Tnpo3-expressing retrovirus and cultured the cells on OP9 feeder cells for 7 d. As a control, we transduced the mutant pro-B cells with a Tnpo1-expressing retrovirus. By flow cytometric analysis, we observed a fourfold increase in the frequency of CD19$^+$ cells relative to vector transduced *Tnpo3*$^{fl/fl}$ pro-B cells (Fig. 4E). In Tnpo1 transduced cultures, we found a decrease in the frequency of CD19$^+$ pro-B cells. No effects of Tnpo1 or Tnpo3 overexpression were detected in parallel experiments with *Tnpo3*$^{+/+}$ pro-B cells (data not shown). Tnpo1 and Tnpo3 show only 20% sequence identity and 35% sequence similarity. Therefore, we assessed the specificity of Tnpo3:EBF1 interaction by coimmunoprecipitation of HA-tagged Tnpo3 and Tnpo1 with FLAG-tagged EBF1^{wt} and EBF1^{E271A} in transfected HEK293 cells (Fig. 4F). We detected a coimmunoprecipitation only with Tnpo3 and EBF1^{wt}, confirming the specificity of this interaction.

To gain some insight into the molecular basis of the B cell differentiation defect in the bone marrow of *Tnpo3*$^{fl/fl}$*mb1*^{Cre} mice, we performed an RNA-seq analysis of FACS-sorted B220$^+$CD43$^+$HSA$^+$ pro-B cells. In *Tnpo3*-deficient pro-B cells, this analysis identified 1214 down-regulated and 1224 up-regulated genes with a significant and more than twofold change of expression relative to *Tnpo3*$^{+/+}$ pro-B cells (Fig. 5A). Gene ontology (GO) term analysis revealed the down-regulation of genes important for early B cell differentiation and proliferation and V(D)J recombination and the up-regulation of genes associated with T cell differentiation, proliferation, and NK T cell differentiation (Fig. 5B). The down-regulated genes included the transcriptional regulators of B cell differentiation *Ebf1*, *Pax5*, and markers of early stages of the B lineage (Fig. 5C; Supplemental Table S4). Moreover, *Tnpo3*-deficient pro-B cells displayed an up-regulation of T lineage and NK lineage genes, including *Notch1*, *Ccr9*, *Cd28*, *Tcf7*, and multiple members of the *Klr* gene family of NK receptors (Fig. 5C; Supplemental Table S4). The reduced but not abolished expression of B lineage markers and the up-regulation of T and NK lineage genes in the mutant cell population indicate that the cells may have acquired a mixed lineage identity.

By overlapping the deregulated genes with EBF1^{wt} ChIP-seq data of pro-B cells, we found that 549 out of 1214 down-regulated and 490 out of 1224 up-regulated genes are bound by EBF1 (Fig. 5D). We also determined the overlap of EBF1 occupancy and deregulation of genes in *Tnpo3*$^{fl/fl}$ pro-B cells with the deregulation of genes observed in OP9-DL1 cocultured EBF1^{E271A}-expressing CD19$^-$CD25$^-$ progenitor cells. The 19 genes that were commonly up-regulated in *Tnpo3*$^{fl/fl}$ and EBF1^{E271A} cells included genes encoding the T lineage determinant TCF1, Notch1, thymus-specific chemokine receptors, and components of the T cell receptor (Fig. 5E; Supplemental Table S5). Commonly down-regulated genes included *Fcer2a*, encoding CD23; *Mtor*, encoding a central component of the MTORC1 and MTORC2 signaling complexes; and *Rragd*, coding for a guanine nucleotide-binding protein that plays an important role in the cellular response to amino acid availability through regulation of MTORC1 signaling (Sancak et al. 2008). Finally, we overlapped the EBF1-occupied and *Tnpo3*$^{fl/fl}$-deregulated gene sets with gene sets of tamoxifen-treated *Ebf1*$^{fl/fl}$*RERT*^{Cre} pro-B cells (Treiber et al. 2010b). Notably, 24% (133 out of 549) of EBF1-occupied and *Tnpo3*$^{fl/fl}$-down-regulated genes were also down-regulated in *Ebf1*-deficient pro-B cells, and 11% (54 out of 490) of *Tnpo3*$^{fl/fl}$-up-regulated genes were up-regulated in *Ebf1*-deficient cells (Supplemental Table S6). Thus, the *Tnpo3* deficiency in early B lineage cells results in transcriptome changes that partially overlap with transcriptome changes in *Ebf1* deficiency.

Discussion

Here, we show that Tnpo3 interacts with EBF1 via glutamic acid 271 in the IPT domain of EBF1, and we found that Tnpo3 enhances EBF1 function in B cell programming. The finding that EBF1^{E271A}-expressing progenitors showed a reduced potential of B cell programming relative to EBF1^{wt}-expressing cells in cocultures with OP9-DL1 feeders suggests that Tnpo3 may be required for EBF1 function in conditions of Notch signaling. Although the exact molecular interplay between EBF1 and Notch1 is still unknown, these lineage determinants have been proposed to counteract each other in establishing B cell versus T cell developmental programs (for review, see Rothenberg 2014). This antagonism is reflected by the observation that EBF1-induced B lineage differentiation was less efficient when progenitors were cocultured with OP9-DL1 feeder cells as compared with cocultures with OP9 feeders. Moreover, the analysis of clonal switch cultures, in which multipotent progenitors were individually placed on OP9 or OP9-DL1 feeders and transferred onto the opposite feeder after varying times, indicated that DL1-exposed progenitors retain their developmental plasticity until the down-regulation of *Ebf1* and *Pax5* and the up-regulation of *Tcf7* and *Gata3* (Taghon et al. 2005). In our experiments, in which ectopic EBF1^{wt} is expressed via GFP-bicistronic retroviruses, prolonged Notch signaling is not sufficient to induce T cell differentiation at the expense of B cell differentiation, suggesting that Notch-induced repression of the endogenous *Ebf1* gene is important for enabling activation of the T lineage program. This scheme is consistent with the observation that the conditional *Ebf1* inactivation or a reduced *Ebf1* gene dosage in mice results in the up-regulation of *Tcf7* and alternative lineage markers in pro-B cells, reflecting an impaired lineage identity (Lukin et al. 2011; Nechanitzky et al. 2013). In B lineage-promoting OP9 cocultures, we observe modest changes in B cell-specific gene expression that are enhanced in the nonpermissive conditions of OP9-DL1 cocultures. In this context, EBF1^{E271A}-expressing progenitors also initiate T cell programming, as evidenced by the surface expression of CD25 and the activation of T lineage genes. However, the E271A mutation is not sufficient for allowing a complete lineage

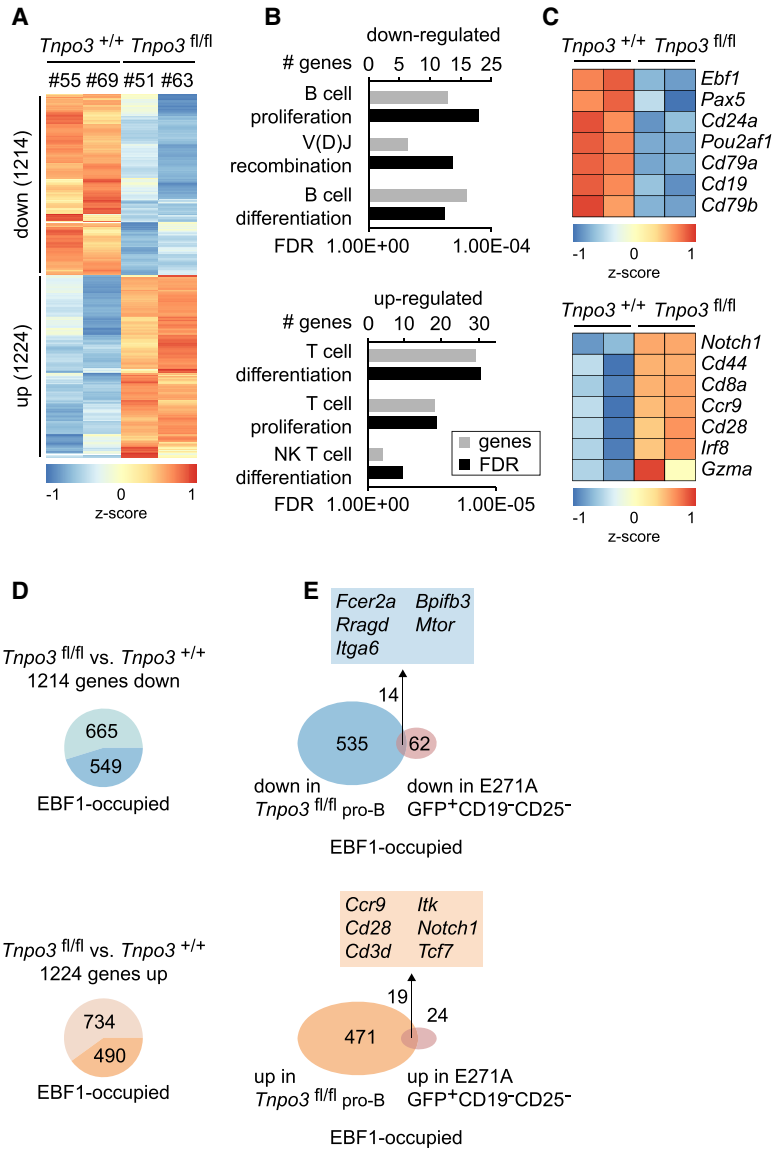

Figure 5. Transcriptome of *Tnpo3*$^{fl/fl}$*mb1*^{Cre} pro-B cells shows impaired B lineage identity. (*A*) Heat map of the 2438 genes significantly deregulated ($q < 0.05$) in B220$^+$CD43$^+$HSA$^+$ pro-B cells from the bone marrow of *Tnpo3*$^{fl/fl}$*mb1*^{Cre} mice (#51 and #63) relative to corresponding cells from *Tnpo3*$^{+/+}$*mb1*^{Cre} mice (#55 and #69). Clusters with twofold down-regulated and up-regulated genes are indicated. (*B*) Gene ontology (GO) term analysis of down-regulated (*top*) or up-regulated (*bottom*) genes. Numbers of genes and FDR are indicated by gray and black bars, respectively. (*C*) Heat maps depicting the down-regulation of representative B lineage genes (*top*) and up-regulation of representative T and NK lineage genes (*bottom*) in *Tnpo3*$^{fl/fl}$*mb1*^{Cre} pro-B cells relative to *Tnpo3*$^{+/+}$*mb1*^{Cre} pro-B cells. (See Supplemental Table S4 for a complete list of genes.) (*D*) Relative numbers of down-regulated (*top*) and up-regulated (*bottom*) genes that are occupied by EBF1 as determined by ChIP-seq analysis in pro-B cells (Treiber et al. 2010b; Boller et al. 2016; Li et al. 2018). (*E*) Overlap of EBF1-occupied genes that are down-regulated (*top*) or up-regulated (*bottom*) in *Tnpo3*$^{fl/fl}$*mb1*^{Cre} pro-B cells and in CD19$^-$CD25$^-$ EBF1^{E271A}-expressing HSPCs cocultured with OP9-DL1 for 7 d. Representative genes are indicated. (See Supplemental Table S5 for a full list of genes.)

switch because the level of TCF1 expression in EBF1^{E271A}-expressing progenitors, determined by intracellular FACS analysis, is significantly lower than that observed in vector transduced cells. Thus, the EBF1^{E271A}-expressing cells may acquire a mixed lineage identity in T cell-promoting conditions.

The relationship of the in vitro cell culture and the in vivo situation is limited. The analysis of the B lineage-specific deletion of the *Tnpo3* gene in mice indicates that Tnpo3 is required for normal differentiation of pre-pro-B cells from pro-B cells. However, the differentiation block in *Tnpo3*$^{fl/fl}$*mb1*^{Cre} is less severe than that found in *Ebf1*$^{fl/fl}$*mb1*^{Cre} mice (György et al. 2012) but stronger than that detected in the in vitro cultures. In the *Tnpo3* mutant mice, CD19$^+$ cells are detected at reduced frequencies in the periphery, which could be a consequence of the incomplete block of early B cell differentiation. A preliminary analysis of a *Cd21*^{Cre}-mediated deletion of *Tnpo3* indicated that the loss of Tnpo3 in transitional B cells has no significant effect on the frequencies of mature B cells in the spleen (data not shown). Therefore, Tnpo3 may have a function in early but not late stages of B cell differentiation. A specific function of Tnpo3 in early stages of T cell differentiation has been reported in mice and zebrafish (Iwanami et al. 2016).

The transcriptome analysis of Tnpo3-deficient CD19$^+$ pro-B cells indicated that the mutant cells show a down-regulation but not a loss of *Ebf1* and *Pax5* expression and a reduced expression of multiple B lineage genes encoding components of the pre-BCR and BCR signaling pathways. Moreover, *Tnpo3* mutant cells also show a down-regulation of genes associated with cell adhesion and migration. Notably, in *Tnpo3* mutant pro-B cells, we detected a marked up-regulation of genes specific for T and NK lineages, suggesting that the cells have gained alternative lineage identities. The comparison of transcriptome changes in *Tnpo3*$^{fl/fl}$*mb1*^{Cre} pro-B cells and in tamoxifen-treated *Ebf1*$^{fl/fl}$*RERT*^{Cre} pro-B cells indicated

a 24% and 11% overlap of down-regulated and up-regulated EBF1-occupied genes, respectively. This partial but significant overlap may reflect common and distinct functions of Tnpo3 and EBF1. Tnpo3 has been implicated in post-transcriptional gene regulation and pre-mRNA splicing (Kataoka et al. 1999; Lai et al. 2000; Iwanami et al. 2016). In *Tnpo3*-deficient pro-B cells, however, we did not detect any major changes in splicing patterns of deregulated genes (data not shown), suggesting that EBF1-associated Tnpo3 may have distinct transcriptional functions.

The mechanism by which the loss of Tnpo3 interaction with EBF1^{E271A} leads to impaired B cell programming in conditions of Notch signaling is still unclear. The ChIP-seq analysis of EBF1^{wt} and EBF1^{E271A} indicated that chromatin binding of EBF1 is largely unaffected by the E271A mutation. However, Tnpo3 may affect a direct or indirect interaction of EBF1 with the Notch transcription complex, which consists of the intracellular domain of Notch bound to the RBPJ/CBF1 transcription factor and coactivators of the Mastermind-like (MAML) family (for review, see Bray 2016). In Epstein-Barr virus (EBV)-infected human B cells, the EBV nuclear antigen-2 (EBNA2) associates with RBPJ- and EBF1-occupied sites with a preference for cobound sites (Portal et al. 2013; Lu et al. 2016; Glaser et al. 2017). Recently, the interplay of EBV oncoproteins and EBF1 function was extended by studies of a mouse model of EBV-mediated and MYC-driven lymphomagenesis in which EBNA3A was found to phenocopy EBF1 function in promoting transformation and inhibiting plasma cell differentiation (Sommermann et al. 2020). Finally, an interplay between Notch and EBF1 has been reported in Notch-driven T-ALL cells in which a Notch-responsive 3′ *MYC* enhancer mediates oncogenic *MYC* overexpression that is maintained after a long-term treatment of the cells with a γ-secretase inhibitor via a compensatory gain of *EBF1* expression (Zhou et al. 2022). In our EBF1 ChIP-seq analysis of cells with induced NICD expression, we did not detect a significant enrichment of the RBPJ-binding motif in EBF1 peak regions; however, we cannot exclude that EBF1 interacts with RBPJ-occupied sites over large distances. Therefore, it will be interesting to examine whether the interplay between Notch and EBF1 in transformed cells may be affected by the interaction with Tnpo3.

Although EBF1 and Notch functions appear mutually exclusive and do not intersect in bone marrow lymphopoiesis, two peripheral B cell populations encounter or even depend on Notch ligands. First, EBF1-expressing marginal zone B cells require signaling by Notch2 for their development and maintenance (Tanigaki et al. 2002; Saito et al. 2003; Lechner et al. 2021). Moreover, conditional deletion of the Notch ligand gene Delta-like 1 results in a complete disappearance of marginal zone B cells in mice (Hozumi et al. 2004). Notch signaling also leads to the proteasomal degradation of the E2A transcription factor (Nie et al. 2003; Zhang et al. 2013), whereby low levels of E2A can be compensated for by the function of EBF1 (Quong et al. 2004; Seet et al. 2004). Although we can detect some marginal zone B cells in the spleen of *Tnpo3*$^{fl/fl}$*mb1*^{Cre} mice, the overall decrease in the number of B cells makes it difficult to assess a role of Tnpo3 in the generation of marginal zone B cells. Second, thymic B cells colocalize with Notch ligand-expressing thymic epithelium at the cortico–medullary junction (Miyama-Inaba et al. 1988; for review, see Perera and Huang 2015). This B cell population has a highly activated phenotype and plays a functional role in central T cell tolerance and autoimmunity (Perera et al. 2013; for review, see Castañeda et al. 2021). Thymic B cells share with B1a cells the expression of CD5 and with follicular B cells the surface expression of both CD21 and CD23 (Miyama-Inaba et al. 1988; Perera et al. 2013). The origin of thymic B cells is still controversial. However, their developmental pathway intersects with Notch signaling. In Notch-deficient mice, the thymic B cell population expands but also changes its cellular phenotype by losing its typical CD5 expression and acquiring the expression of AA.1, a marker of bone marrow B cell precursors (Wilson et al. 2001; Feyerabend et al. 2009). Therefore, it will be interesting to examine whether the EBF1:Tnpo3 interaction may influence the development of B cells that depend on or are exposed to Notch signaling. As EBF1 and Tnpo3 appear to have both overlapping and distinct functions, future studies will have to involve mice carrying the Ebf1^{E271A} alleles that abrogate specifically the interaction of EBF1 with Tnpo3.

Materials and methods

Mice

E16.5–E18.5 *Ebf1*$^{-/-}$ embryos were obtained by crossing *Ebf1*$^{+/-}$ mice (Lin and Grosschedl 1995). *mb1*^{Cre} *Tnpo3*$^{fl/fl}$ mice were obtained by crossing *mb1*^{Cre} and *Tnpo3*$^{fl/fl}$ mice (Hobeika et al. 2006; Iwanami et al. 2016). Mice were maintained under specific pathogen-free conditions in the laboratory animal facility of the Max Plank Institute of Immunobiology and Epigenetics. Mouse experiments were carried out according to the guidelines of the Federation of European Laboratory Animal Science Association (FELASA) and following legal approval of the animal committee in Freiburg.

Cell culture and retroviral transduction

c-kit^{+} *Ebf1*$^{-/-}$ HSPCs were purified and cultured as previously described (Boller et al. 2016). To inhibit Notch signaling, HSPCs were treated with 10 µg/mL DAPT (Abcam 208255-80-5). Retroviral transduction was performed as previously described (Treiber et al. 2010b) and HSPCs were sorted for retroviral expression 16 h after transduction. The inducible NICD1 expression system was established by sequentially transducing *Ebf1*$^{-/-}$ HSPCs with pMYs-rtTA-tdTomato, pRetroX-Tight-Pur-FLAG-NICD1 (Clontech), and pMYs-EBF1wt-FLAG-Strep-IRES-GFP or pMYs-EBF1E271A-FLAG-Strep-IRES-GFP and selecting cells accordingly. For NICD1 induction, cells were treated with 1 µg/mL doxycycline for 6 h. A-MuLV transformed pro-B cells of *Ebf1*$^{fl/fl}$ *RERT*^{Cre} mice were obtained from Györy et al. (2012) and cultured as previously described. For siRNA-mediated knockdown, cells were transduced with the pSIREN-RetroQ retroviral expression plasmid (Clontech) expressing siRNAs targeting *Tnpo3* (GGGAGT TTCGAATGAGAGTGT) or *Gfp* (GCACAAGCTGGAGTA CAACTA).

Flow cytometry

Single-cell suspensions from bone marrow, spleen, or fetal liver were subjected to red blood cell lysis. Bone marrow and fetal liver cells were stained with fluorescently labeled antibodies against CD43 (S7), CD45R/B220 (RA3-6B2), HSA (M1/69), and BP1 (BP-1). Splenic cells were stained using antibodies against CD19 (6D5), CD21 (7G6), and CD23 (B3B4). *Ebf1*$^{-/-}$ HSPCs were stained with antibodies against CD19 (1D3), CD19 (6D5), CD25 (PC61), BP1 (BP-1), and CD45R/B220 (RA3-6B2). Intracellular staining of EBF1 and TCF-1 was performed using the eBioscience Foxp3/transcription factor staining kit (Invitrogen) and anti-FLAG (M2) and anti-TCF-1 (C63D9) antibodies. Antibodies are listed in the Supplemental Material.

Immunoblot analysis

Immunoblot analysis was performed using the following antibodies: Actin (Sigma A2066), EBF1 (clone 6G6; Dr. Kremmer, Helmholtz Zentrum München), FLAG (clone M2; Sigma-Aldrich F1804), GAPDH (clone 6G5; Calbiochem CB1001), HA (clone 3F10; Roche 11867423001), Lamin A (Sigma L1293), and Tnpo3 (clone 3152C2a; Abcam ab54353). Antibodies are listed in the Supplemental Material.

Coimmunoprecipitation and SILAC-MS

Coimmunoprecipitation and SILAC-MS of endogenous EBF1 and Strep–Tactin pull-downs were performed as previously described (Yang et al. 2016).

mRNA sequencing analysis

Total RNA was isolated from cells using the RNeasy micro or mini kit (Qiagen). RNA quality was determined on a fragment analyzer. Paired-end libraries were prepared using the SMART-seq v4 ultralow-input RNA library preparation kit (Takara 634893) or the NEBNext low-input RNA library preparation kit for Illumina (NEB E6420) and sequenced on a HiSeq 2500 or HiSeq 3000 system.

ChIP-seq analysis

EBF1 ChIP was performed as previously described (Boller et al. 2016). For spike-in normalization, murine pro-B cells were mixed with the EBF1-expressing human B cell lymphoma cell line BJAB in a 10:1 ratio to simultaneously immunoprecipitate murine and human EBF1 with the same antibody. Bioinformatic spike-in normalization using the human EBF1 ChIP signals was performed as described in the Supplemental Material. Sequencing libraries were prepared using the NEBNext Ultra II DNA library preparation kit for Illumina (NEB E7645) and sequenced on a HiSeq 3000 system.

Data availability

Data sets generated in this study are available as a superseries in the GEO database GSE201531.

Competing interest statement

The authors declare no competing interests.

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Author contributions: M.B. and S.B. designed and performed experiments. M.B., S.R., N.Z., and P.C. conducted bioinformatic analysis. G.M. conducted mass spectrometric analysis. N.I. established and provided *Tnpo3*$^{fl/fl}$ mice. T.B. supervised N.I. and gave advice. R.G. conceived and supervised the study. M.B. and R.G. wrote the manuscript with input from all authors.

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