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cDNA-library testing identifies transforming genes cooperating with c-myc in mouse pre-B cells

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While c-myc often contributes to the generation of B cell transformation, its transgenic overexpression alone does not lead to full transformation of B-lineage cells. Synergistically acting second genes must cooperate. Here, we constructed doxycycline-inducible cDNA-libraries from pre-B cell mRNA. These libraries were retrovirally transduced as single copies into single cells and overexpressed in fetal-liver-derived c-myc-overexpressing pre-B cell lines. We scored transformation by survival and/or expansion of differentiating B-lineage cells in vitro and in vivo. Only one double c-myc/cDNA-library-expressing cell line was found in less than 5 × 10^6 library-transduced pre-B cells surviving and expressing a cDNA-library-derived transcript in vitro. This transcript was identified as a shortened form of the *Exosc1* gene, encoding the RNA exosome complex component CSL4. Transplantations of double c-myc/Exosc1 short-form- or c-myc/Exosc1 full-length-transgenic cells into Rag1$^{-/-}$ mice resulted in survival, differentiation to CD19$^+$CD93$^-$sIgM$^+$CD5$^{low/-}$CD11b$^+$ mature B1 cells and, surprisingly, also vigorous expansion in vivo. Strikingly, after transplantations of c-myc/cDNA-library pre-BI cells the frequencies of double-transgenic pre-B cells and their differentiated progeny, expanding in vivo to heterogeneous phenotypes, was at least ten-fold higher than in vitro. In a first analysis *Ptprcap, Cacybp, Ndufs7, Rpl18a,* and *Rpl35a* were identified. This suggests a strong influence of the host on B-cell transformation.

Keywords: B-lineage cell transformation · cDNA-library overexpression · c-myc · Exosc1

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Introduction

Genes that contribute to cell cycle entry, proliferation, differentiation, and apoptosis or survival of pre-BI lymphocytes and their differentiated progeny are expected to be contained in their mRNA and, hence, in cDNA-libraries generated from their mRNA. Deregulation and overexpression of some of these genes are expected to result in partial transformation on the way to malignant states, in which B-lineage cells show decreased apoptosis and/or proliferation, possibly at defined stages of B-cell development. By transducing pre-BI cell lines with such genes, we can follow the consecutive development towards their progeny, both in vitro and, after transplantation, in vivo.

Many B cell leukemias develop in successive steps, in which genetic and microenvironmental factors change the expression of genes regulating proliferation, differentiation and survival [1–3]. Deregulated overexpression of these oncogenes can fully transform cells at distinct stages of cellular development, and generate long-lived, expanding cells in vivo which also might be able to proliferate and/or survive as cell lines in vitro [4]. Early studies have

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already indicated, that full transformation requires at least two functionally cooperating oncogenes, one providing anti-apoptotic, the other pro-proliferative, or tumor suppressor factors-disruptive activities [5–7].

The effects of c-myc on cell cycle entry-enhancement, proliferation-activation, B-lineage cell-deregulation, and genome destabilization have been analyzed extensively [8–14]. We have previously transduced wild-type pre-BI cell lines, proliferating on stromal cells (OP9) in the presence of IL7 [15], with SIN-retroviral vectors containing doxycycline-inducible forms of cell-cycle enhancing c-myc and anti-apoptotic pim-1 [4]. When wild-type, nontransduced pre-B cell lines are removed from the stimulatory action of IL7 and OP9 stromal cells, they cease to proliferate, differentiate to pre-BII, and $sIgM^+$ immature B cells, and rapidly enter apoptosis [15]. Doxycycline-controlled overexpression of c-myc in pre-B cells enhanced entry into cell cycle, but did not inhibit normal apoptosis, while pim-1 overexpressing cells showed minimally reduced apoptosis, but did not proliferate. Differentiation of the nontransduced, as well as c-myc- or pim-1-single transduced pre-BI cells to IgM^+ B cells remained normal, i.e. was induced by the removal of OP9 stroma and IL7 in vitro, or by transplantation into Rag-deficient hosts. By contrast, joint doxycycline-controlled overexpression of c-myc together with pim-1 (c-myc/pim-1) allowed differentiation to IgM^- pre-BII-like cells. These cells survived and expanded by proliferation in vitro and in vivo. This expansion was reversed, when doxycycline was removed, resulting in normal development to IgM^+ B cells without further deregulated proliferation or survival [4].

These studies indicated that single oncogene-transgenic cells could be suitable targets to test B cell-lineage- or B-lymphoma-derived cDNA-libraries for second, cooperating oncogenes. If we chose pim-1 single transduced cells, we would screen for proliferation-enhancing c-myc-like activities, while we would test for pim-1-like, survival-enhancing activities, if we used the c-myc-overexpressing cells. Here, we report our search for cDNA genes with anti-apoptotic, c-myc-complementing actions, in c-myc-transduced pre-BI cells and their progeny, induced by differentiation in vitro and in vivo.

Since the establishment of techniques for full-length cDNA cloning [16] and later full-length cDNA-library generation [17] in the 1980s, cDNA-libraries became a powerful tool for identification of new oncogenes [18–20]. Beside their identification, cDNA-library screens allow the detection of alterations within the exonic DNA, splice alterations or mutation of already known and unknown transforming genes.

We hypothesize that deregulated overexpression of many genes, might have yet unknown transforming impacts on cellular development. Therefore, we generated a cDNA-library from wild-type in vitro proliferating pre-BI cells. We chose the Gateway® cloning to transfer the cDNA-libraries to a doxycycline-inducible expression vector system (Supporting Information Fig. 1). This system allows cloning without restriction enzymes, which might corrupt some of the genes within the library [21].

We overexpressed this cDNA-library in c-myc-overexpressing pre-BI cells and screened for cells which survive in the absence of growth supporting stromal cells and IL7, i.e. undergo transformation by the cooperating actions of the cDNA-library-derived genes and c-myc.

By synergistic overexpression of c-myc and cDNA-library-derived genes in pre-BI cells, we detected one in 5×10^6 cells that long-term survived without OP9/IL7. However, when these 5×10^6 c-myc/cDNA-library-transduced pre-BI cells were tested in vivo by transplantation into immunodeficient $Rag1^{-/-}$ mice, we detected five to tenfold higher numbers of double transduced cells, indicating a major host influence in the process of transformation. These transformed cells did not only survive, but were also found to expand and to differentiate toward progressed B-lineage stages in vivo. The structural identification of this first set of in vitro and/or in vivo-transforming genes from cDNA-libraries identified a wide spectrum of proteins, which appear to destabilize their normal functions, when they are deregulated and overexpressed.

Results

C-myc-transgenic pre-B cells as targets for transductions with cDNA-libraries

The cDNA expression library was generated in three steps. First, the cDNA-library double strand fragments were reverse transcribed from mRNA of in vitro cultivated proliferating pre-BI cells [15]. The cDNA was fused to short nucleotide sequences (attB1 and attB2), which are essential for following recombination procedures into different vector systems (Supporting Information Fig. 1A). Second, the cDNA was recombined into an entry vector (Supporting Information Fig. 1B). Third, the entry library was transferred into a retroviral destination vector, forming an expression library. As destination vector a self-inactivating (SIN) retroviral vector was used (Supporting Information Fig. 1B) [4]. In this vector, gene expression is controlled by a tetracycline-response-element (TRE) and is induced in the presence of doxycycline (TetON) [22]. Finally, the quality of both cDNA-libraries was assessed by restriction enzyme analysis and Sanger sequencing followed by Blast and Alignment analysis (Supporting Information 2).

A previously established fetal-liver-derived pre-BI cell line by our laboratory (4) was used to screen for genes contained in our cDNA library that cooperate with c-myc in pre-BI cell transformation. This pre-BI cell line has previously been transduced with a vector encoding the reverse-transactivator (rtTA) and has been selected for high rtTA expression [4] (rtTA-pre-BI). To track doxycycline-inducible c-myc-transduced cells in vitro and in vivo, we constructed a polycistronic vector, expressing c-myc and GFP, connected by a F2A peptide (Supporting Information Fig. 3A), [24–26]. The c-myc-F2A-GFP (c-myc-GFP) plasmid was retrovirally transduced into fetal-liver derived rtTA-pre-BI cells generating a c-myc-GFP pre-BI cell line. Upon addition of doxycycline, c-myc-GFP expression was confirmed on protein levels or by fluorescence cytometry (Supporting Information Fig. 3B–D). The c-myc-GFP pre-BI cell line was further transduced with

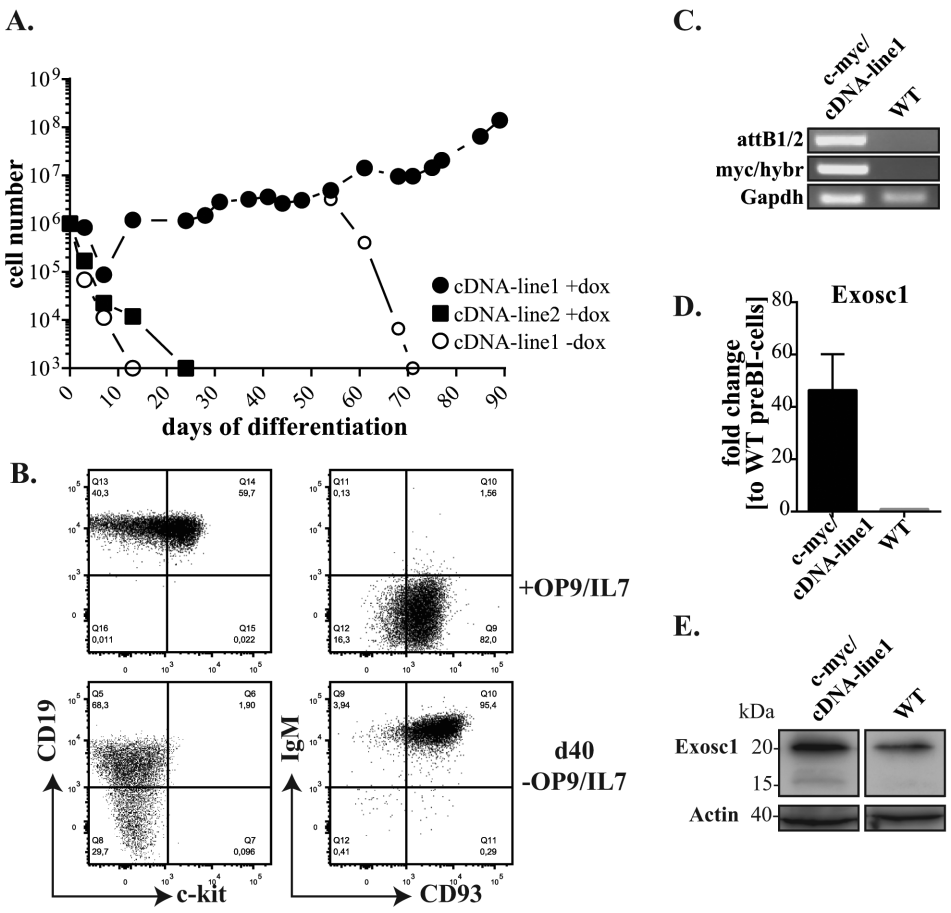

Figure 1. Identification of a shortened transcript of the *Exosc1* gene cooperating with c-myc in pre-BI cell transformation in vitro. (A) Survival and differentiation analysis of c-myc and cDNA-library-line1 and 2 overexpressing pre-BI cells after OP9 and IL7 deprivation in vitro in the presence (closed circles) or absence (open circles) of doxycycline was measured by counting living cells using a hemacytometer and Trypan blue exclusion. (B) Changes in expression of CD19, c-kit, CD93 and IgM by c-myc/cDNA-library overexpressing pre-BI cells before and 40 days after OP9/IL7 deprivation were measured by flow cytometry. Gating strategy see Supporting Information Fig. 6. (C) RT-PCR with primers specifically binding attB1 and attB2 regions (attB1/2) flanking the expression cDNA-library derived gene of interest (myc/hybr, Gapdh = controls). (D) qRT-PCR analyses using Exosc1 gene as internal primer. Data are shown as fold change to wild-type pre-BI cells, presented as mean ± SEM of 5 samples. (E) Detection of Exosc1sf in cDNA-library-line1 cells by Western Blot. Actin = loading control. Data shown are representative of 3–5 (A-D) and 1 (E) independent experiment(s).

tertiary doxycycline-inducible constructs containing either no inserts (empty vector) or the cDNA library (Supporting Information Fig. 3A).

To determine the minimum cell number, necessary to detect oncogene-driven transformation, we double-transduced c-myc-GFP pre-B cells with pim-1 [4] and empty vectors (Supporting Information 3E&F), mixed in different ratios. We differentiated the cells in the absence of OP9 and IL7. While c-myc and pim-1 double-transgenic pre-BI cells proliferated and differentiated to surrogate light chain expressing [23] pre-BII cells, single, non-transduced or empty vector expressing cells died [4].

For the first 28 days of differentiation, 50 c-myc/pim-1 expressing cells were sufficient to detect oncogene-driven transformation of pre-B cells by growth. However, to detect oncogene-driven transformation beyond 28 days, 5×10^3 c-myc/pim-1 double-expressing cells were necessary to be detectable (Supporting Information 3E).

Transformation of c-myc-transgenic pre-BI cells with cDNA expression library-derived genes in vitro

We transduced 1×10^6 c-myc-GFP pre-BI cells each with retroviruses containing cDNA-library-derived genes at a multiplicity of infection (MOI) of one. Subsequently, we differentiated these cells in the absence of OP9 and IL7. In five separate experiments, only one c-myc/cDNA library cell line (c-myc/cDNA-line1) survived for 20 days and beyond (Fig. 1A). Within 20 days of OP9/IL7 deprivation, the $GFP^+CD19^+CD93^+c\text{-}kit^+sIgM^-$ pre-BI cells had differentiated toward $CD19^+CD93^+c\text{-}kit^-sIgM^+$ immature-like B cells (Fig. 1B). When doxycycline was removed from culture, the cells did not survive, indicating that the transformation was dependent on overexpression of c-myc and the cDNA library-derived gene (Fig. 1A).

By RT-PCR using attB1/2 anchor primers (Fig. 1C) we amplified one single cDNA-library-derived gene product, which we

identified by Sanger sequencing and subsequent Blast analysis as a short-form of the *Exosc1* gene (Exosc1sf) (GenBank: KX371790). The Exosc1sf transcript was truncated at the C-terminal end and lacked the stop-codon (Supporting Information 4). The wild-type Exosc1 encodes the protein exosome complex component CSL4 [29].

By quantitative real-time PCR using gene internal primers, the Exosc1 transcript levels were 42-fold upregulated in c-myc/cDNA-line cells compared to wild-type pre-BI cells (Fig. 1D). In addition to the wild-type, full-length CSL4 protein of 21 kDA, we detected a shortened, Exosc1sf encoded, CSL4 protein of ~15 kDa in Exosc1sf-overexpressing pre-BI cells by Western Blot (Fig. 1E). The cell line c-myc/cDNA-line1 was changed to c-myc/Exosc1sf.

C-myc/Exosc1sf immature-like B cells expand and differentiate to mature B1 cells in vivo

Within 40 days of in vitro culture without OP9 and IL7, c-myc/Exosc1sf pre-BI cells differentiated to immature-like B cells but not beyond (Fig. 1B). To investigate, whether these c-myc/Exosc1sf cells would have the potential to differentiate towards later B-lineage stages, we transplanted 5×10^6 day 40 differentiated c-myc/Exosc1sf immature-like B cells into doxycycline-fed, immunodeficient ($Rag1^{-/-}$) mice (Fig. 2A). Four weeks after transplantation, the spleen of doxycycline-fed c-myc/Exosc1sf mice increased in size (Fig. 2B). GFP^+ cells were detected in the bone marrow and spleen of all recipients (Fig. 2C). These data indicate that the combined overexpression of c-myc and Exosc1sf leads to an oncogenic transformation of differentiating pre-B cells in vitro, which can also be propagated in vivo. Since the transplanted cells increased in numbers, i.e. expanded, it suggested that the host provided stimulating influence and increased efficiency of B-cell expansion.

We further tested, whether mutagenic insertions of retroviral vectors into c-myc-GFP-transduced pre-BI cells, could account for the development of transformed cells. Hence, pre-BI cells, double-transduced with c-myc-GFP and an empty vector (c-myc/empty vector) (Supporting Information Fig. 3A&B), were generated, and their expansion in vivo after transplantation was monitored (Fig. 2C). One hundred-fold lower numbers of GFP^+ cells were detected, predominately as pre-BI cells, in bone marrow and spleen, when compared with the numbers of GFP^+ cells, detected in mice transplanted with immature-like B cells transduced with c-myc and Exosc1sf. We conclude that, at most 1% of all GFP^+ cells might have developed from transplanted cells with mutagenic insertions of the retroviral vector, and that 99% of these GFP^+ cells developed as a result of the dual overexpression of c-myc and cDNA-library-derived Exosc1sf. This also confirmed previous results [4] showing that overexpression of c-myc alone is insufficient to induce transformation, ligand-independent survival or proliferation of B-lineage cells in vitro and in vivo.

The transferred c-myc/Exosc1sf immature B cells were predominately detected as $CD19^+c\text{-}kit^-sIgM^+CD93^+$ immature B cells and as $CD19^+ckit^-CD93^-sIgM^+IgD^-CD21^-CD23^-CD5^{low/-}CD11b^+$ B-lineage cells (Fig. 2C). As we would expect [30], these data indicate that these fetal-liver-derived cells differentiated toward mature B1 cells in vivo. Furthermore, slightly increased IgM but no IgG concentrations were detected in sera of doxycycline-fed mice 4 weeks after transplantation of c-myc/Exosc1sf cells (Fig. 2D). Doxycycline-fed mice transplanted with c-myc/Exosc1sf immature-like B cells died on average 60 days after transplantation (Fig. 2E). Control mice, transplanted with c-myc/Exosc1sf-transduced immature-like B cells in the absence of doxycycline, did not expand GFP^+ cells in the bone marrow or spleen, nor did their sera contain elevated levels of IgM or IgG. These data indicate that the expansion of GFP^+ B-lineage cells in vivo and the elevated levels of IgM are a result of c-myc/Exosc1sf co-expression pre-BI cells and their differentiated progeny.

When we isolated GFP^+ c-myc/Exosc1sf transplanted cells from bone marrow and spleen of doxycycline-fed mice and cultured them in vitro in the absence or presence of doxycycline, the cells did not expand and survived at least for 14 days (Fig. 2F). This indicates that the transplanted host contributed essential expansion- and differentiation-stimulating influences on the c-myc/Exosc1sf cells.

Transforming activity of the full-length Exosc1 transcript

To test, whether the full-length Exosc1 could also have transforming activity, we transduced c-myc-GFP pre-BI cells with a retroviral doxycycline-inducible plasmid encoding full-length Exosc1 (Exosc1fl) cDNA (Supporting Information Fig. 5). Simultaneous overexpression of c-myc and Exosc1fl, again, induced anti-apoptotic activity in vitro after OP9/IL7 deprivation, though less strongly than the truncated form (Fig. 3A).

Four weeks after transplantation of c-myc/Exosc1fl pre-BI cells into doxycycline-fed $Rag1^{-/-}$ recipients, GFP^+ cells accumulated in the bone marrow and spleen (Fig. 3B), however in tenfold lower numbers compared to transplantations with c-myc/Exosc1sf transformed immature B cells (Fig. 2C). Most GFP^+ cells gave rise to a heterologous population of $CD19^+c\text{-}kit^+CD93^+sIgM^-$ pre-BI cells, $CD19^+c\text{-}kit^-CD93^+sIgM^+$ immature-like B cells and $CD19^+ckit^-CD93^-sIgM^+CD21^-CD23^-CD5^{low/-}CD11b^+$ mature B1 cells (Fig. 3B). Reduced IgM concentrations and no IgG were detected in serum of doxycycline-fed mice transplanted with c-myc/Exosc1fl pre-B cells (Fig. 3C).

We conclude that the full length Exosc1 gene can cooperate with c-myc to transform B-lineage cells, though less efficiently than the short-form Exosc1.

Pre-BI cell pools co-overexpressing c-myc and cDNA-library genes expand in vivo to different stages

In previous experiments we found, that the in vivo environment of transplanted $Rag1^{-/-}$ recipients exerts proliferative influence

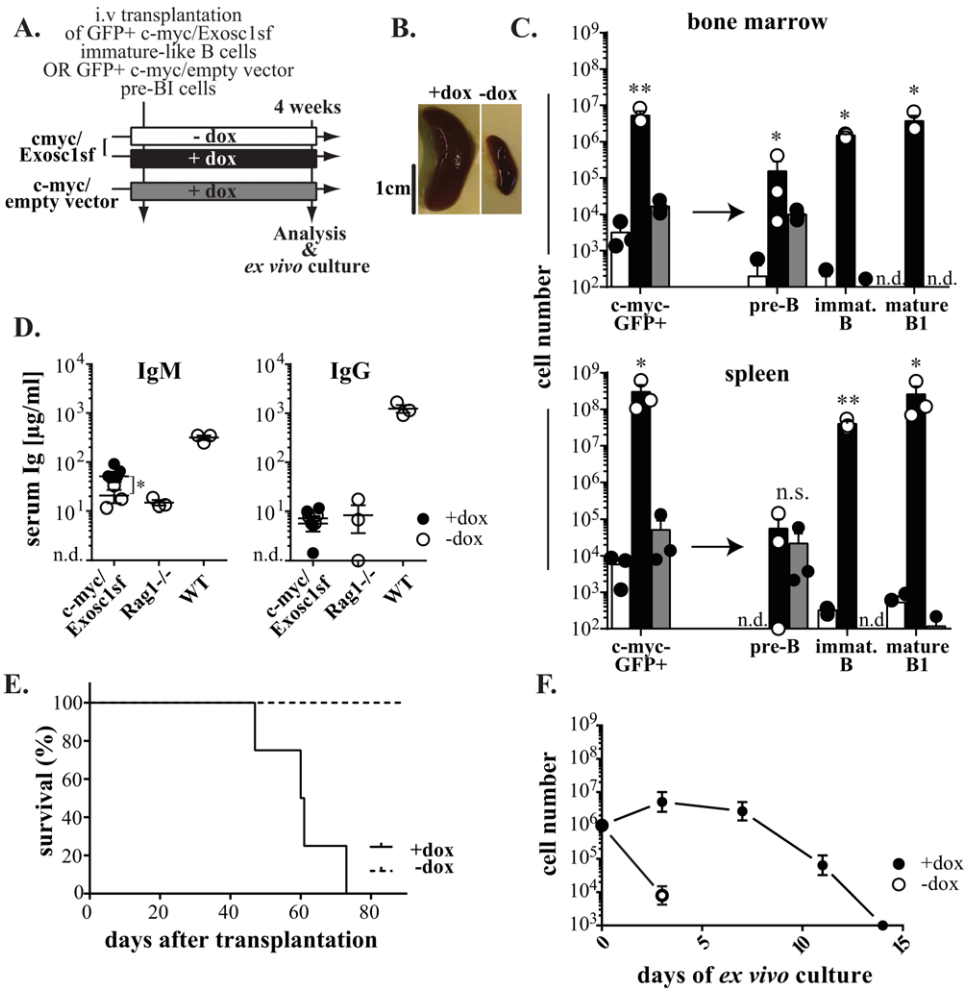

Figure 2. C-myc/Exosc1sf immature-like B cells expand and differentiate to mature B1 cells in vivo. (A) Transplantation schedule of GFP$^+$ c-myc/Exosc1sf immature-like B cells into doxycycline-fed (black bars) and non-fed (open bars) immunodeficient (Rag1$^{-/-}$) mice. As control, one group of doxycycline-fed Rag1$^{-/-}$ mice was transplanted with GFP$^+$ pre-BI cell lines overexpressing c-myc and an empty, doxycycline-inducible vector (c-myc/empty vector). (B) Splenomegaly detected in doxycycline-fed immunodeficient recipients (+dox, closed circles) compared to the nondoxycycline-fed control group (-dox, open circles). (C) FACS analysis of bone marrow (1 hind leg) and spleen-derived GFP$^+$ c-myc transgenic B-lineage cells 4 weeks after transplantation of c-myc /Exosc1fl pre-BI cells into immunodeficient Rag1$^{-/-}$ mice. The c-myc-GFP$^+$ cells were subgated (arrow pointing right) on CD19$^+$ckit$^+$CD93$^+$ pre-BI cells, CD19$^+$ckit$^-$CD93$^+$sIgM$^+$CD21$^-$CD23$^-$ immature B cells, and CD19$^+$ckit$^-$CD93$^-$sIgM$^+$CD21$^-$CD23$^-$CD5$^{low/-}$CD11b$^+$ mature B1 cells. Dots indicate cell numbers detected in individual mice. n.d. = viable cells not detected. (D) ELISA of IgM and total IgG in sera of transplanted mice 4 weeks after transplantation. Rag1$^{-/-}$ and wild-type (WT) C57BL/6 = controls, n.d. = Ig secretion not detected. (E) Survival of doxycycline-fed c-myc/Exosc1sf transplanted mice (solid line; $n = 10$) compared to their control group which received no doxycycline (dashed line; $n = 5$) (Kaplan–Maier analysis). (F) Ex vivo differentiation culture of bone marrow derived GFP$^+$ cells in the presence (closed circles) or absence (open circles) of doxycycline. (A–F) Data are shown as mean ± SEM of $n = 3$ and 5 mice/group/experiment and are representative of two independent experiments. Statistical significance was determined by 1-way ANOVA (C) and two-tailed unpaired *t*-test (D); $^*p < 0.05$, $^{**}p < 0.005$.

on the in vitro nonexpanding c-myc/Exosc1sf and c-myc/Exosc1fl cells. Therefore, we next screened c-myc/cDNA-library double-transduced pre-B cells for additional cDNA-library-derived genes, which might be susceptible to host stimulation for in vivo expansion after transplantation.

In a second round of c-myc-GFP pre-BI cell-transfection, new doxycycline-inducible c-myc/cDNA-library double-transgenic pre-BI-cell lines were generated. Again, 5×10^6 of the c-myc/cDNA-library gene-transduced pre-BI cells were induced to differentiate in vitro upon OP9/IL7 deprivation, while another 5×10^6 of these cells were directly transplanted into Rag1$^{-/-}$ recipients (Fig. 4A), both in the presence of doxycycline. As seen before, simultaneous overexpression of c-myc and cDNA-library genes had a weak anti-apoptotic, and no proliferation-inducing effects on the collection of double-transgenic pre-BI cells (Fig. 4B). The cells did not survive beyond day 20 after removal of OP9/IL7.

Surprisingly, and in contrast to transplantations of c-myc/Exosc1sf and c-myc/Exosc1fl-transgenic B-lineage cells, not only pre-BI, immature and matureB1 cells were detected. The mice transplanted with c-myc/cDNA-library genes-transduced pre-BI cells were found to contain additionally, heterogeneous pools of cells resembling CD19$^{+/low}$CD138$^+$IgM$^+$ TACI$^-$MHC$^{high/+}$ plasmablasts and CD19$^{low/-}$CD138$^+$IgM$^-$TACI$^+$MHC$^{low/-}$ plasma cells (Fig. 4C).

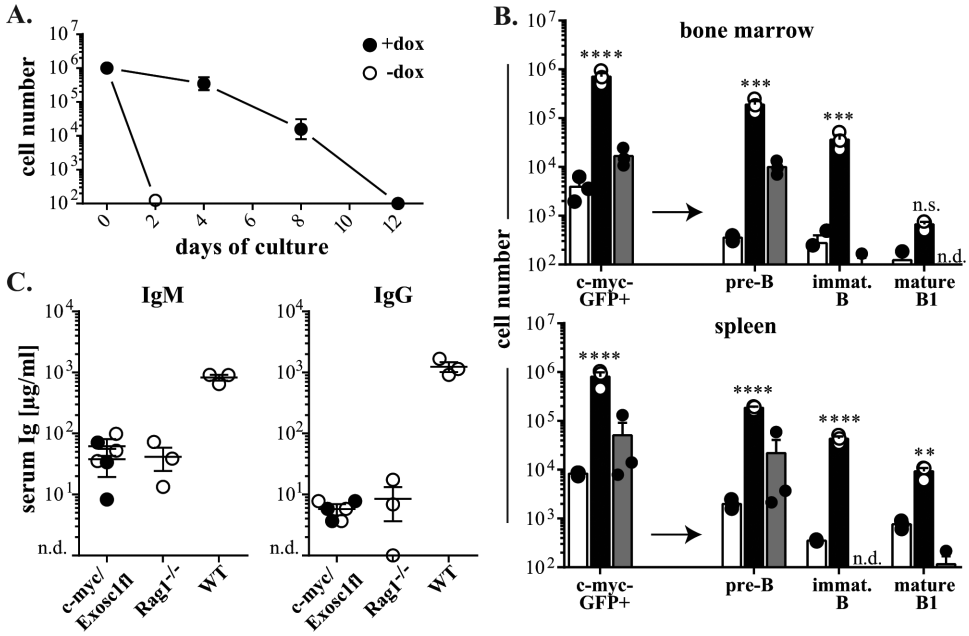

Figure 3. C-myc/Exosc1fl pre-BI cells expand and differentiate to mature B cells in vivo. (A) Differentiation of c-myc/Exosc1fl overexpressing pre-BI cells upon OP9/IL7 deprivation in the presence (closed circles) or absence (open circles) of doxycycline in vitro, enumerated by counting living cells using a hemacytometer and Trypan blue exclusion. (B) FACS analysis of bone marrow (1 hind leg) and spleen derived GFP$^+$ B-lineage cells 4 weeks after transplantation of c-myc/Exosc1fl (black bars) or c-myc/empty vector (grey bars) pre-BI cells into doxycycline-fed (closed symbols) or non-fed (open symbols) immunodeficient Rag1$^{-/-}$ mice. The c-myc-GFP$^+$ cells were sub-gated (arrow pointing right) on CD19$^+$ckit$^+$CD93$^+$ pre-BI cells, CD19$^+$ckit$^-$CD93$^+$sIgM$^+$CD21$^-$CD23$^-$ immature B cells and CD19$^+$ckit$^-$CD93$^-$sIgM$^+$CD21$^-$CD23$^-$CD5$^{low/-}$CD11b$^+$ mature B1 cells. n.d. = viable cells not detected. (C) ELISA of IgM and total IgG in doxycycline-fed transplanted mice 4 weeks after. Rag1$^{-/-}$ and wild-type (WT) C57BL/6 = controls, n.d. = Ig secretion not detected. Data are shown as mean ± SEM of $n = 3$ mice/group/experiment and are representative of two independent experiments. Statistical significance was determined by 1-way ANOVA; $^{**}p < 0.005$, $^{***}p < 0.0005$, $^{****}p < 0.0001$, n.s. = statistically not significant.

Spleen-derived GFP$^+$ cells of some, but not all of these mice expressed increased amounts Blimp1, a master regulator of terminal B-cell development to plasma cell differentiation [31], while other mice harbored GFP$^+$ cells expressing AID transcripts, which is restricted to germinal center B cells [32] (Fig. 4D). By contrast, Blimp1 and AID transcripts were not found in spleens from doxycycline-fed mice transplanted with c-myc/Exosc1sf or c-myc/Exosc1fl cells. This suggests, that the c-myc/Exosc1 transformed cells were all arrested in vivo as sIgM$^+$ mature B1 cells and did not differentiate toward later B-lineage stages, while some of the cells transfected with cDNA genes of the library developed plasmablasts and plasma cells.

Furthermore, in some, although not all mice reconstituted with c-myc/cDNA-library cells, elevated levels of IgM, but not IgG, were detected in the sera (Fig. 4E). The heterogeneous gene expression patterns and Ig serum levels coincide with the heterogeneous FACS phenotypes of transplanted GFP$^+$ c-myc/cDNA-library cells.

After transplantations of c-myc/cDNA-library-overexpressing pre-BI cells, heterogeneous pools of differentiated B-lineage cells developed in vivo. These heterogeneous pools of double-transduced cells differentiated either to immature B and mature B1 cell stages, or proceeded to later, germinal center-like or plasmablast/plasma-like cell stages. Our data indicate that this B-lineage heterogeneity might be a result of different sets of cDNA-library-derived genes that cooperate with c-myc in cell transformations. Single cell cloning of the double-transduced B-lineage cells ex vivo, followed by re-transplantation, will have to be done to resolve this heterogeneity of transformed cells, and to determine, which cDNA-library-genes induce differentiation to mature B cells, and which other genes allow further differentiation to plasmablasts and plasma cells.

Identification of cDNA-library genes cooperating with c-myc in B-cell transformation in vivo

Next, GFP$^+$ cells from bone marrow were isolated ex vivo and analyzed for abundance of cDNA-library-derived genes by RT-PCR. Several different transcripts of different sizes were amplified from the RNA of GFP$^+$ bone marrow cells from each of the transplanted mice (Fig. 5). This indicates that the pool of transplanted, in vivo expanded c-myc/cDNA-library cells expressed several different cDNA-library-derived genes in each of the transplanted recipients. Five cDNA-library-derived PCR products were sequenced (Fig. 5). They were identified as *Rpl18a*, *Rpl35a Ndufs7*, *Cacybp,* and *Ptprcap*. All sequences were full-length and without mutations.

Based on the results described above, we conclude that the frequency of genes which support c-myc in B cell transformation in vivo is much higher than in vitro, thus suggesting that the

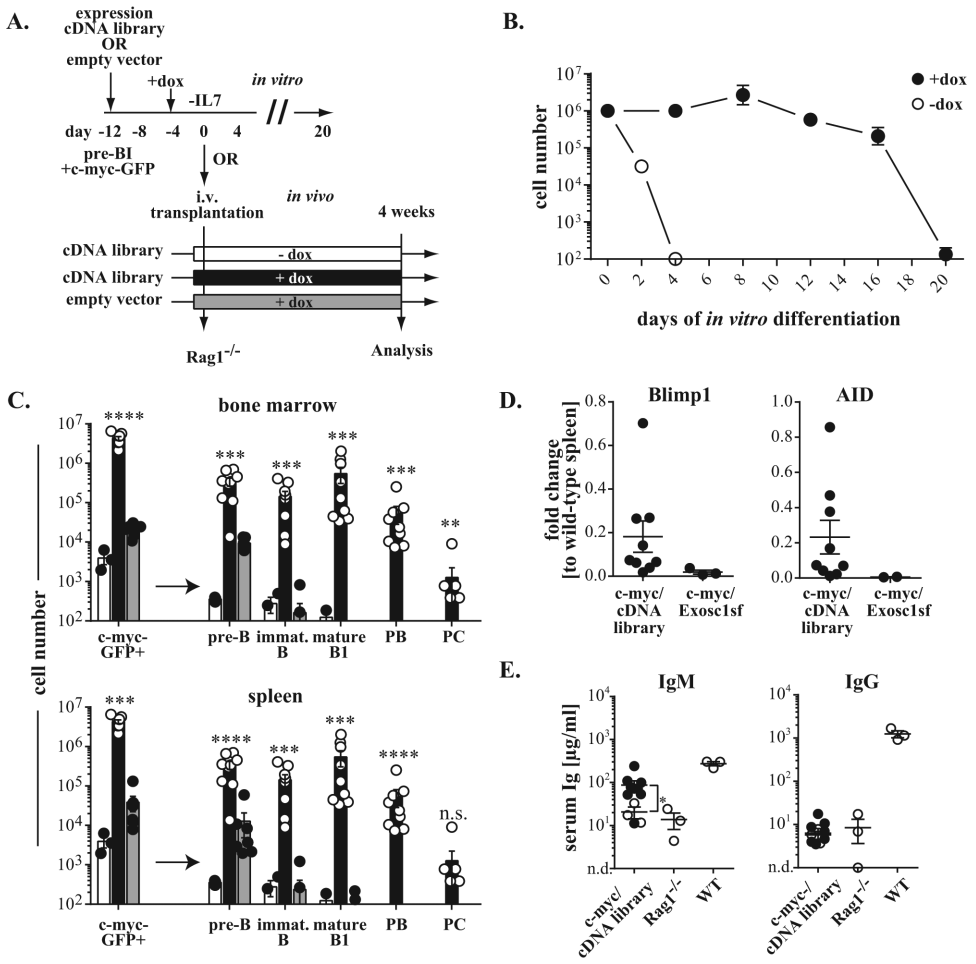

Figure 4. Transplantation of c-myc/cDNA-library overexpressing pre-BI cells. (A) In vitro culture and transplantation schedule of c-myc/cDNA-library transgenic pre-BI cells. (B) Differentiation analysis of c-myc/cDNA-library overexpressing pre-BI cells upon OP9/IL7 deprivation in the presence (closed circles) or absence (open circles) of doxycycline in vitro. (C) FACS analysis of bone marrow (1 hind leg) and spleen derived GFP$^+$ c-myc/cDNA-library cells 4 weeks after transplantation into doxycycline-fed (black bars) or doxycycline-non-fed (open bars) Rag1$^{-/-}$ mice. As control, one group of doxycycline-fed Rag1$^{-/-}$ mice was transplanted with GFP$^+$ pre-BI cell lines overexpressing c-myc and an empty, doxycycline-inducible vector (c-myc/empty vector, gray bars). The c-myc-GFP$^+$ cells were subsequently gated for pre-B cells (CD19$^+$c-kit$^+$CD93$^+$IgM$^-$), immature B cells (CD19$^+$c-kit$^-$CD93$^+$IgM$^+$), mature B1 cells (CD19$^+$ckit$^-$CD93$^-$sIgM$^+$CD21$^-$CD23$^-$CD5$^{low/-}$CD11b$^+$), plasmablasts (CD19$^{+/low}$CD138$^+$MHCII$^+$ IgM$^+$) and plasma cells (CD19$^{low/-}$CD138^{hi}MHCII$^-$IgM$^-$TACI$^+$). Gating strategy see Supporting Information Fig. 7. (D) qRT-PCR of GFP$^+$ cell-derived RNA from spleens of c-myc/cDNA-library pre-BI cells or c-myc/Exosc1sf immature-like B cell chimeras. Data are presented as fold change of mRNA levels relative to wild-type spleen cells (E) ELISA of IgM and total IgG in sera of transplanted mice 4 weeks after transplantation of c-myc/cDNA-library overexpressing pre-BI cells. Rag1$^{-/-}$ and wild-type (WT) C57BL/6 = controls, n.d. = Ig secretion not detected. (B-E) Data are shown as mean ± SEM of $n = 6$ and 9 and 3 (control) mice/experiment and are representative of two independent experiments. Statistical significance was determined by 1-way ANOVA (C) and two-tailed unpaired t-test (E); $^{**}p < 0.005$, $^{***}p < 0.0005$, $^{****}p < 0.0001$.

host provides a transformation-stimulating environment for the development of malignant B cells

Discussion

An extended analysis of one cDNA-library-derived gene with transforming activity in vitro and in vivo has identified a shortened transcript variant of the *Exosc1* gene [33–35].

The wild-type form of Exosc1 is ubiquitously expressed [27, 28] and encodes the protein exosome complex component 1 (CSL4), a core component of the RNA exosome complex which is essential for 3' mediated mRNA degradation, processing and quality control [29].

Since the short-form was not found to be a product of alternative splicing, and did not contain a translation termination codon it poses the question, how such an RNA could be translated into a short protein form, that we detected in Western Blots (Fig. 2). When a message without a stop codon is freed from its association with ribosomes, the growing polypeptide chain behind it could also be freed in this "nonstop decay" reaction. Future experiments should determine, which of the "nonstop decay" mechanisms [36] could operate in c-myc/Exosc1sf-overexpressing pre-BI cells and their progeny to allow the expression of a truncated, stop codon

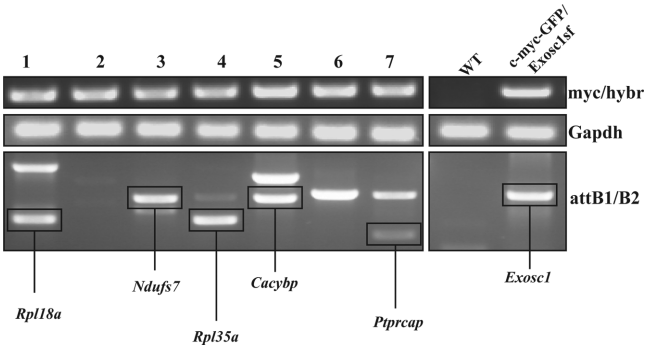

Figure 5. Identification of cDNA-library-derived genes. RT-PCR of GFP$^+$ cells derived from bone marrow of c-myc/cDNA-library transplanted mice. Products were amplified using attB1/B2 cDNA-library specific primers. Numbers indicate one individual mouse, WT = wild-type bone marrow (negative control), c-myc/Exsc1sf = positive control. Gapdh = loading control. Data shown represent two independent experiments performed with 9 and 6 mice, respectively.

missing CSL4 protein, and how the truncated protein, or the full-length protein, disturbs the functions of the exosome.

The RNA exosome consists of a catalytically inactive, barrel-shaped core composed of nine subunits (six subunits form a PH-ring, which is assembled only in the presence of three cap proteins) and one additional unit harboring catalytic activity [37]. The RNA exosome complex component CSL4, is one of the three cap proteins and contains three domains: N-terminal RPL27 providing contact with the PH-ring, a central S1-domain binding mRNA substrate and essential cofactors, and a C-terminal zinc-ribbon-like domain essential for mRNA decay [33, 38, 39]. The S1-domain and the zinc-ribbon-like domain have been found to be dispensable for exosome assembly. Thus, the zinc-ribbon-domain-defective CSL4 protein, produced by Exosc1sf, could still assemble in the exosome, bind RNA, but be unable to degrade mRNA.

In B-lineage cells, overexpression of this shortened CSL4 protein would compete with the endogenously expressed wild-type CSL4 for the binding to the PH-ring and in fact, might replace it. Hence, mRNAs could not be degraded at normal rates, but their concentrations in the transduced cells would increase, leading to enhanced synthesis of proteins. The increased concentration of those proteins, which stimulate survival and/or proliferation of B-lineage cells, would affect increased longevity and expansion.

We have also seen, that the wild-type full-length Exosc1 transcript encoding a full-length CSL4 protein, with an intact zinc-ribbon-like domain, transforms B-lineage cells in vitro and in vivo, indicating that even the full-length CSL4 could interfere with proper mRNA degradation, when overexpressed in B-lineage cells. Under these circumstances, high levels of CSL4 proteins might interfere with the proper formation of the RNA exosome complex, thereby reducing RNA decay activity. Again, this would lead to increased mRNA concentrations in the cell, and result in enhanced translation of, among others, anti-apoptotic and proliferation-stimulating proteins. In addition, high concentrations of free CSL4 proteins might bind mRNAs, inhibiting their proper delivery to the exosome and, thus, their degradation, again with the result of, increased translation.

Our results offer fascinating possibilities to study functions of the exosome complex in situations, where individual components of the complex are defective and/or overexpressed.

Transplantations of c-myc/Exosc1sf, c-myc/Exosc1fl and c-myc/cDNA-library genes indicate that the B-lineage cells not only survive, but also expand by proliferation in vivo. It suggests that the host provides growth- and differentiation-promoting influences. It has long been known that leukemic B-lineage cells, which expand vigorously in vivo and which, in the case of mouse plasmacytomas [40], can be easily transplanted [41], resist proliferation in vitro and rapidly die in cultures. Hence, the host contributes essential stimulation to B-cell leukemias for in vivo proliferation of these malignant cells. The same differences have been observed with human B-cell leukemias. Tissue cultures containing selective cytokines, feeder layer and additional nutritional requirements, which are essential for the establishment and cloning of human malignant lymphoma cell lines, have improved conditions to establish plasmacytoma and lymphoma cell-lines [42, 43]. Overexpression of Exosc1sf, which we show to convey long-term survival to mouse B-lineage cells, in human B-cell leukemias, might further be of help to establish in vitro cell lines from such primary ex vivo tumor cells.

Our in vivo screenings have further identified five cDNA-library-derived genes (*Rpl18a*, *Rpl35a Ndufs7*, *Cacybp,* and *Ptprcap*) that potentially transform B-lineage cells, in cooperation with c-myc. One of the five identified cDNA genes, *Cycybp*, in fact is a c-myc target gene [44]. C-myc is predicted to target and regulate around 11–15% genes of the genome [45, 46]. It is possible, that the c-myc target genes could be preferred cooperating oncogenes in the c-myc-transduced pre-BI cells. Therefore, the identification of a much higher number of cDNA genes would be necessary. However, we do not think that our method for finding cooperating oncogenes selects c-myc targets, since we overexpress nontransformed, i.e. "healthy", in vitro proliferating pre-BI cells. Second transforming, malignancy-propagating genes in in vivo development of B-lineage malignancies might well be selected not only for their function to be transforming (by different types of mutations), but also be selected by deregulated c-myc expression acting via myc-target sequences for myc-sensitivity of their expression. This is not the case in our experiments. Hence, our transformation protocols are likely not selecting for such myc-sensitivity, as an in vivo development of B-lineage tumors might do.

Our results show that single oncogene (e.g. c-myc-) transduced pre-B cells can be used to screen cDNA-libraries for proto-oncogene-mediated cooperating second activity that, when overexpressed, promote development, survival and expansion of B-lineage cells. Their activities are reminiscent of pleiotropically acting mutations in epigenetic modifiers and anti-apoptotic genes that are introduced early in common precursors of follicular lymphomas [47]. Many oncogenes have been previously characterized in B-lymphomas and multiple myelomas with survival- or proliferation-promoting activities of B-lineage cells. Surprisingly, our screen detects a multitude of genes, which were, so far, not considered to be proto-oncogenes. This opens large fields of

further investigations into normal and deregulated actions of many functions in a B-lineage cell.

Materials and methods

Cell culture

RtTA-transduced pre-BI cells, OP9 stroma cells and Plat-E packaging cell line (Life Technologies) were cultivated as previously described [4, 48]. Differentiation of pre-BI cells was induced upon removal of OP9 stroma cells and IL7, or of IL7 alone.

Transduction of pre-BI cells

Retroviral vectors were transiently transfected into Plat-E cells using Lipofectamine (Life Technologies) as previously described [4]. The gene expression was achieved by addition of 1.5 μg/mL doxycycline into the culture medium.

Vectors

Doxycycline-inducible, "TreTight"-TetOn-controlled [49] SIN retroviral vectors encoding c-myc-, pim-1-, the cDNA-library, Exosc1fl or no insert (empty vector) were constructed as reported [4] (Supporting Information Fig. 1B and Fig. 3A). The c-myc used in our studies is a mouse-human hybrid sequence that allows differential detection of endogenous and transduced c-myc in RT-PCR analyses. For simultaneous expression of c-myc and GFP (c-myc-GFP), a polycistronic vector was constructed, which expressed both genes from one transcript, connected by sequences encoding the 22 amino acid-long 2A peptide (Supporting Information 3, A), a cis-acting hydrolase element that allows skipping between the conserved glycine and proline [24–26]. After processing, the expression of this vector yields a c-myc protein with one additional amino acid from the 2A peptide at the amino-terminal end, and a longer than normal GFP protein with 20 amino acids from the 2A peptide at the carboxy-terminal end.

For the generation of a doxycycline-inducible expression vector the attR flanked ccdB gene and Chloramphenicol resistance gene (Cm^R)was amplified from destination vector (pDEST14, Life Technologies) and inserted into the linearized vector. pENTR™ 1A (Life Technologies) was used for the generation of the entry library. Sequence of inserted genes was verified by Sanger sequencing (Eurofins MWG Operon Sequence).

Western blot

Western Blot analysis were performed as previously described [4]. Exosc1 was detected using anti-mouse Exosc1 (EPR13525, Abcam).

RNA and mRNA purification

Total RNA was purified using TRIzol® Reagent (Life Technologies). For cDNA-library generation RNA of 2×10^8 fetal-liver derived pre-BI cells cultivated on OP9 stroma and IL7 was purified followed by mRNA isolation using the FastTrack® MAG mRNA Isolation Kit (both from Life Technologies) according to the manufacturer's instructions. For RT- and qPCR RNA of 5×10^5-1×10^7 cells was extracted. The mRNA concentration and purity was estimated by spectrophotometry (NanoDrop 1000, Peqlab).

Generation of a cDNA library

The full-length cDNA-library was generated according to the SuperScript® Full Length cDNA-library Construction Kit II (Life Technologies) protocol. If not noted otherwise, all reagents were supplied with the kit. Between 5 and 10 μg mRNA were used the production of a cDNA-library containing 1.0×10^7 to 1.2×10^7 primary cDNA clones in *E.coli*. The plasmid DNA from bacterial colonies was purified using Plasmid Miniprep Kit (Sigma, Qiagen, Hilden; Germany) and the heterogeneity was confirmed by BsrGI (entry library) or XhoI (expression library) (New England Biolabs, Ipswich MA, USA) digestion. Sequence analysis were performed with following primers: Entry library 5′-TGTAAAACGACGGCCAGT-3′ and 5′-CAGGAAACAGCTATGACC-3′,

Expression library 5′-TGATAGAGAACGTATGTCGAGGTAG-3′ and 5′-TATCCAGCCCTCACTCCTTCTC-3′. The sequence results were blasted in Nucleotide BLAST followed by sequence alignment with the matching homologue mRNA sequence and coding DNA sequence (CDS) derived from Nucleotide database (ncbi.nlm.nih.gov/nuccore) using SeqMan Pro software (DNAStar®, Madison, WI, USA). Functional annotation analysis was performed using DAVID functional annotation tool (https://david.ncifcrf.gov). For detailed cDNA-library qualification analyses see Supporting Information 2.

Semiquantitative and quantitative real-time RT-PCR

For cDNA preparation, equal amounts of RNA and dilutions thereof were used for each condition. Transcription of RNA into cDNA was performed using SuperScript III reverse transcriptase (Life technologies) and oligodT or random hexamer primers (Thermo Fisher Scientific, Waltham, MA, USA). Amplification of cDNA was performed using primers specific for:

myc/hybr 5′-CGGATTCTCTGCTCTCCTCGAC-3′ and 5′-CGTCGAGGAGAGCAGAGAATCC-3′, Gapdh 5′-TTGAGGTCAATGAAGGGGTC-3′ and 5′-TCGTCCCGTAGACAAAATGG-3′,

attB1/B2 5′-GCCTGGAGAATTCGGATCTAGAT-3′ and 5′-ACCACTTTGTACAAGAAAGTTGGGT-3′

pInsert 5′-TCCTCCCTTTATCCAGCCCT-3′ and 5′-TGTACGGTGGGAGGCCTATA-3′

Exosc1 5′-GGAGCTGTCGTCACCTGTAA-3′ and 5′-GCTCGGATATCTTCTTTGCGG-3′

IgG1 5′-GACCTCTACACTCTGAGCAGC-3′ and 5′-GGGGAAGA TGAAGACAGATGAT-3′

IgM 5′-ACCTGAATGTGTACACCTGCC-3′ and 5′-AGGAAGA TGTCGGCAAAGGAG-3′

AID 5′-TAGTGCCACCTCCTGCTCAC-3′ and 5′-AGGAGGTGA ACCAGGTGACG-3′

Blimp1 5′-TTGGTACACACAGGAGAGAAGCC-3′ and 5′-TAGTGTTTCTGCAGGTGGGC-3′

RT-PCR was performed using Taq DNA Polymerase (Thermo Fisher Scientific), while quantitative RT-PCR was conducted with QuantiTect SYBR Green PCR Kit (QIAGEN) according to manufacturer's protocol.

Flow cytometry

Cells were stained with anti-mouse CD19-BV650 (6D5), B220-BV510 (RA3-6B2), CD93-PerCPC5.5 (AA4.1), CD138-BV421 (281-2), c-kit-AlexaFlour700 (ACK4), IgK-PE (RMK-45), IgG1-bio (RMG1-1), IgG2a-bio (RMG2a-62), IgG2b-bio(RMG2b-1) (BioLegend, San Diego CA, USA) and CD25-PeCy7 (eBio3C7), IgM-APC or –PeCy7 (II/41), TACI-APC (ebio8F10-3) (eBioscience, San Diego CA, USA) in the presence of anti-FCgRII (2.4G2, in-house). Streptavidin-Qdot605 (Molecular Probes) was used to visualize biotin-conjugated primary antibodies. Lymphocates were analyzed using an LSRII FACS (BD Biosciences) in the presence of DAPI (Carl Roth GmbH). Aggregates and doublets were gated out [4]. Acquisition was performed using the DiVa software 6.1 (BD Biosciences). Analysis was performed using the FlowJo software (Tree Star, Ashland OR, USA).

Transplantation of pre-BI and differentiated cells into Rag1$^{-/-}$ recipients

All experiments were performed as already described [4]. All animal procedures were conducted in compliance with the German animal protection laws with the protocol approved by the Landesamt fur Gesundheit und Soziales, Berlin (G0140-11).

ELISA

Immunoglobulin (Ig) levels of serum samples, compared with standard Ig class samples, were determined with Maxisorp ELISA 96-well plates coated with goat anti-mouse IgM or total IgG as prescribed by Southern Biotech, Birmingham AL, USA. Using AP-labeled goat anti-mouse IgM-AP, or total IgG-AP antibody (Southern Biotec) the serum Igs were quantified.

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Abbreviations: **dox**: Doxycycline · **fl**: full-length · **rtTA**: reverse transactivator · **sf**: short form · **TRE**: Tetracycline response element

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