Universidad Autónoma de Madrid

Departamento de Biología Molecular

Tesis Doctoral

“The role of *c-myc* in the transcriptional regulation of B lymphocyte differentiation”

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1. INTRODUCTION

1. Introduction

1.1. c-Myc transcription factor

The *c-myc* proto-oncogene is the cellular homologue of *v-myc*, the transforming gene of the MC29 avian leukaemia virus (Sheiness et al., 1978). Deregulated *c-myc* expression has been demonstrated in many types of human cancers, including Burkitt's lymphoma, myeloid and plasma cell leukaemia, breast carcinoma, cervical carcinoma, small cell lung carcinoma, colon carcinoma, osteosarcoma, and glioblastoma (Spencer and Groudine, 1991; DePinho et al., 1991). Myc family genes (comprising C-, N-, and L-Myc) code for proteins that promote proliferation, growth, and apoptosis, inhibit terminal differentiation and, when deregulated, are greatly involved in the genesis of an extraordinarily wide range of cancers (Grandori et al. 2000).

1.2. c-Myc protein

The gene product encoded by the *c-myc* proto-oncogene is a transcription factor whose carboxy terminus contains three structural domains homologous to domains found in other transcription factors, including a leucine zipper (Zip), a helix-loop-helix motif (HLH), and an adjacent domain rich in basic amino acids (b) (Fig 1) (Blackwell et al., 1990; Landshulz et al., 1988; Murre et al., 1989).

The HLH and Zip motifs mediate protein-protein interaction, and the basic region mediates sequence-specific DNA binding (Blackwell et al., 1990; Landshulz et al., 1988; Murre et al., 1989). The c-Myc amino terminus (aa 1-143) comprises the TAD region, which is required for transcriptional activation and is conserved in all Myc family members. The TAD region includes Myc box I (MBI), which has two important phosphorylation sites (Thr58 and Ser62), associated with activation and protein degradation (Stone et al., 1987; Sears et al., 2004), Myc box II (MBII), needed for interaction with the transformation/transcription domain-associated protein (TRRAP) transcriptional coactivator and critical for most Myc biological functions (Oster et al., 2002; Pelengaris et al., 2002; Freytag et al., 1990), Myc box III (MBIII), involved in

transcriptional repression (Kurland and Tansey, 2008; Herbst et al., 2005) and Myc box IV (MBIV), which contains one of the c-Myc nuclear localisation signals (NLS) and is implicated in modulating DNA binding (Cowling et al., 2006).

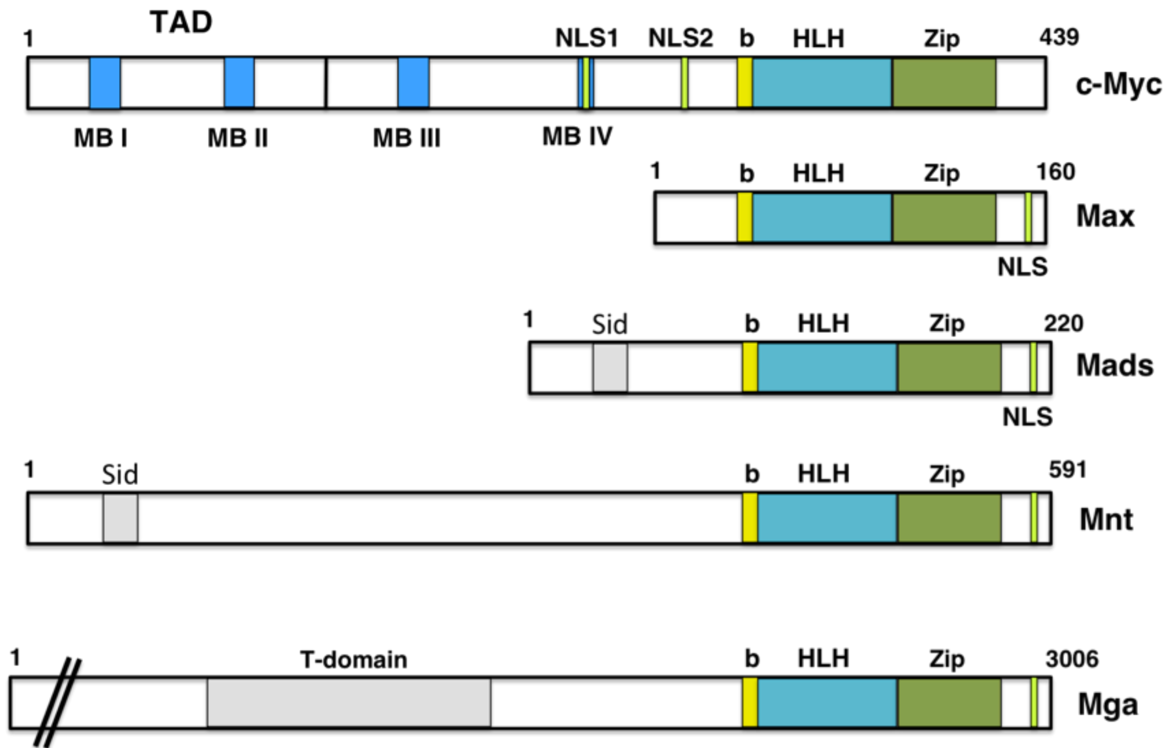


Fig 1. Structure of Myc/Max/Mad network proteins. Functional domains of c-and different bHLHZ transcription factors

1.3. c-Myc regulation

Expression of the *c-myc* gene is under intense regulation at both transcriptional and post-transcriptional levels. c-Myc mRNA has an approximately 20-30 min half-life, related to A- and U-rich sequences in the 3' UTR region (Jones and Cole, 1987; Brewer and Ross, 1988). c-Myc protein bears one of the hallmarks of regulatory proteins; it is an extremely short-lived phosphoproteins that is localised predominantly in the cell nucleus.

c-Myc levels are precisely regulated throughout cell life. c-Myc protein is undetectable in quiescent cells, but after exposure to many types of mitogens, its expression is rapidly upregulated and promotes G0/G1 to S phase transition (Kelly et al., 1983; Campisi et al., 1984). The ubiquitin/26S proteasome pathway controls

regulation of c-Myc protein, which has at least 10 phosphorylation sites (Henriksson et al., 1993). c-Myc phosphorylation has an important role in regulation of its proteolysis, just as in the biological functions. Two main phosphorylation sites, Ser62 and Thr58, are responsible for c-Myc stabilisation and degradation. Whereas phosphorylation at Ser62 stabilises c-Myc protein, phosphorylation at Thr58 leads to its degradation (Hann et al., 2006). In many lymphomas, point mutations at c-Myc Ser62 and Thr58 appear to be linked to Myc transformation activity (Nasi et al., 2001).

1.4. The c-Myc network

The bHLHZ domain of Myc, located at its carboxy terminus, mediates heterodimerisation with the bHLHZ protein Max, to produce a heterocomplex with specific DNA-binding activity (Blackwoodand and Eisenman, 1991; Ayer and Eisenman, 1993; Amati and Land, 1994) (Fig 1).

1.4.1. Max

Max is a small, ubiquitously expressed protein that binds to many bHLHZip proteins (Baudino and Cleveland, 2001). Since the Max protein lacks a TAD domain, Myc is responsible for activating transcription of the target genes (Vervoorts and Larsson, 1999). Myc-Max heterodimers recognise the consensus enhancer box (E-box)-related sequence CACGTG with high affinity, and also bind to a group of other E-box- and non-E-box-related sequences with lower affinity. E-box-bound Myc-Max interacts with the basal transcription apparatus and with complexes that remodel and modify chromatin. Myc-Max heterodimers induce relatively modest activation of transcription (from three- to ten-fold) when the binding site(s) is placed proximal to a minimal TATA-box-containing promoter.

1.4.2. Mad, Mnt and Mxi

Max interacts with several distinct classes of bHLHZ proteins, including the Mad protein family and three recently-described proteins, Mnt, Mxi and Mga. When bound to Max, Mad family members bind E-box sequences and compete for binding with c-Myc/Max heterodimers. Mad-Max, Mnt-Max and Mxi-Max complexes function as transcriptional repressors at E-box-containing promoters and are thought to

antagonise Myc-Max functions (Ayer and Eisenman, 1993; Ayer et al., 1993; Zervos et al., 1993; Amati, 2001).

Mad-Max, Mnt-Max, and Mxi-Max complexes mediate transcriptional repression of their target genes through their alpha-helical amino-terminal domain (SID) (Fig 1). This domain is needed for interaction with the transcriptional corepressors Sin3a and Sin3b and with a multisubunit complex that includes the transcriptional corepressors N-CoR, the histones HDAC1 and 2, and the oncoproteins Ski and Sno, all of which alter chromatin structure and block transcription through deacetylation of histone tails (Alland et al., 1997; Hassig et al., 1997; Laherty et al., 1997, Shinagawa et al., 2000) (Fig 2). In contrast to Max, which is ubiquitously expressed, the Mad-Mnt proteins are induced during terminal differentiation of many cell types.

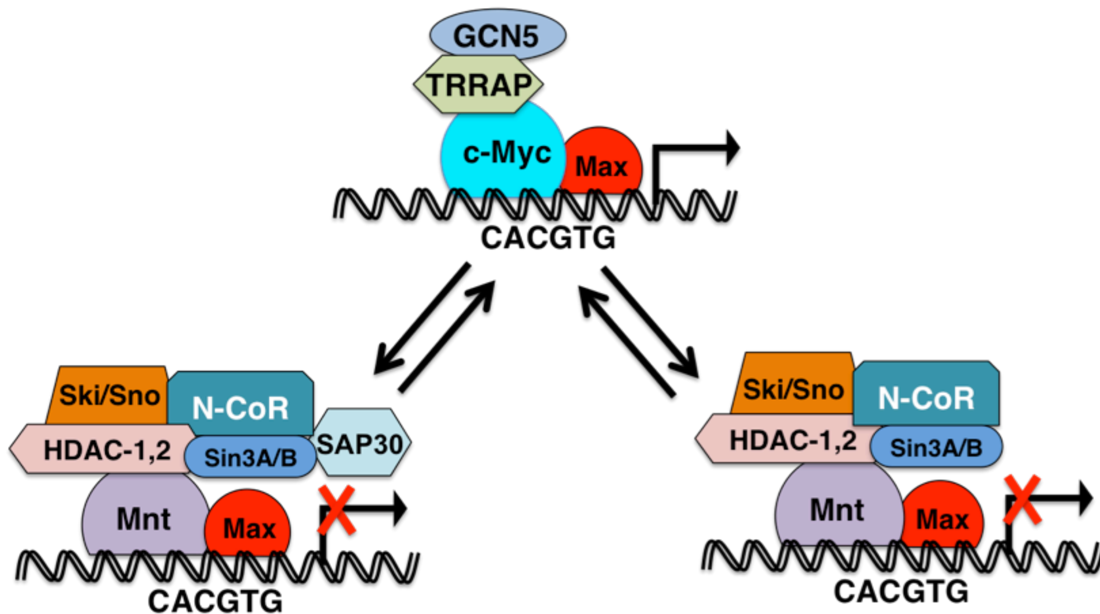


Fig 2. Mechanisms of transcriptional activation and repression by c-Myc network

Another c-Myc-mediated mechanism of trans-repression involves binding to the transcriptional activator Miz-1, which interferes with c-Myc transactivation ability and causes trans-repression of specific Miz-1 target genes (Staller et al., 2001; Wu et al., 2003; Wanzel et al., 2003). One study demonstrated that c-Myc is recruited to the p21 promoter by Miz1. This interaction prevents p21 induction by p53, resulting in the initiation of apoptosis rather than cell cycle arrest (Wu, 2003; Herold et al., 2002;

Seoane et al., 2002). c-Myc acts as a molecular bridge between Miz-1 bound to the p21 promoter and the DNA methylase Dnmt3a to mediate methylation and transcriptional silencing of p21 (Brenner et al., 2005). Another possible mechanism of trans-repression might involve c-Myc interaction with CAAT box-binding proteins such as NF-Y. This appears to be the case with the transcriptional repression of PDGFR-β and perhaps of collagen genes (Hackzell et al., 2002). c-Myc was also shown to repress the proximal promoters of GADD45a and GADD153 by a post-RNA polymerase II recruitment mechanism (Barsyte-Lovejoy et al., 2004).

Mga has a T domain near its amino terminus, which is a conserved DNA and protein-binding motif found in the *Brachyury* family of cell specification transcription factors (Hurlin et al., 1999). It normally represses promoters harbouring T boxes, but activates transcription from E-box when it interacts with Max, switching from a repressor to an activator (Fig 4).

These studies provide evidence that c-Myc can inhibit transcription by directly interfering with other factors that activate gene expression.

1.5. Transcriptional activation

Normally DNA is tightly wound around the histones, and chromatin remodelling is necessary to allow transcription factor access to the regulatory regions of genes. Two mechanisms of chromatin remodelling have been described thus far for c-Myc protein: ATP-dependent remodelling and histone acetylation (Fig 2). During the ATP-dependent chromatin remodelling process, c-Myc interacts with INI1/hSNF5 (Cheng et al., 1999), a component of the SWI-SNF complex that remodels chromatin in an ATP-dependent manner. This reorganisation induces nucleosomes to move along the DNA, enabling access to transcription machinery (Whitehouse et al., 1999). In the case of histone acetylation, c-Myc recruits a coactivator complex containing TRRAP and histone acetyl transferases (HAT) such as GNC5 (McMahon et al., 1998; McMahon et al., 2000) and TIP60 (Campbell et al., 2003), which acetylate H4 and H3 histones, respectively. Acetylation opens up the chromatin structure, allowing enhanced transcription of c-Myc target genes.

1.6. Transcriptional repressor

In addition to its function as a transcriptional activator, which has been broadly studied, c-Myc also acts as a transcriptional repressor. Several genes have been identified as targets for transcriptional repression by Myc, and it is not surprising that many of these genes have roles in cell cycle or growth regulation.

Although most target genes repressed by c-Myc are TATA-less promoters and contain initiator elements (Inr) within the promoter regions, c-Myc is able to mediate repression of genes that lack Inr elements (Gartel and Shchors, 2003). In the case of Inr-dependent repression, c-Myc interacts with Inr-binding transcriptional activators such as TFII-I, YY1 and Miz-1, and forms a DNA-associated complex containing an Inr sequence, leading to inhibition of target gene activation (Henriksson and Luscher, 1996) (Fig. 3). This complex resists further binding of basal transcriptional machinery, and thus prevents transcriptional activation by the Inr (Roy et al., 1993b). A Myc-Max complex binds to the Inr element of the p27 promoter (Yang W et al., 2001). A conserved element in the MBIII region is important for transcriptional

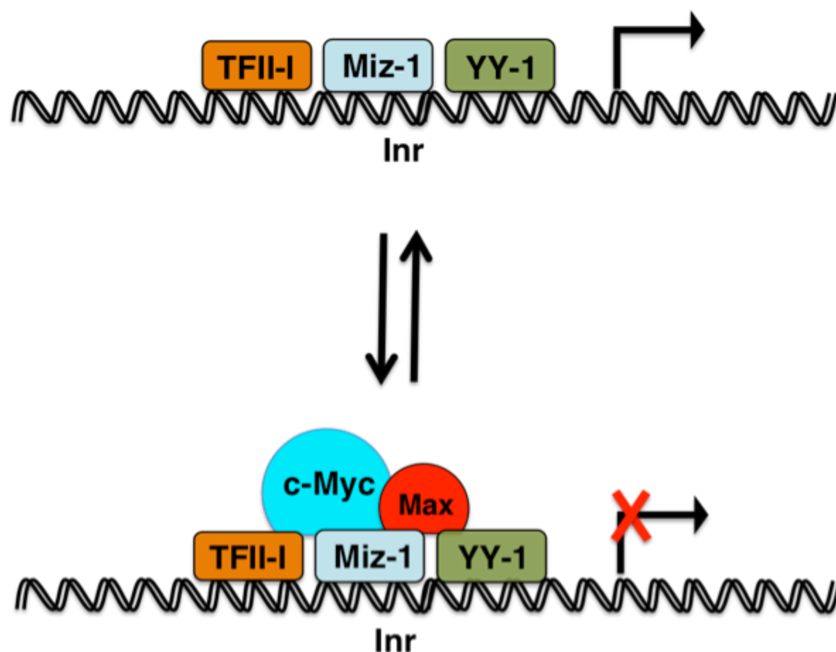


Fig 3. Transcriptional repression mediated by c-Myc through Inr motif

repression of target genes such as Id2 and Gadd153, through recruitment of the HDAC3 histone deacetylases to both gene promoters (Kurland and Tansey, 2008).

In an Inr-independent mechanism, c-Myc binds the transcription factors Sp1 and Sp3 via its central motif, thereby inhibiting their transcriptional activity (Gartel and Shchors, 2003) (Fig. 3). Genes repressed in this way are *gadd45* (Marhin et al.,1997), p15 (Feng et al., 2002) and p21 (Gartel et al., 2001)(Fig 4).

1.7. c-Myc biological functions

c-Myc influences a variety of cell functions, including cell cycle regulation, metabolism, apoptosis, differentiation and adhesion, and deregulated expression participates in tumorigenesis.

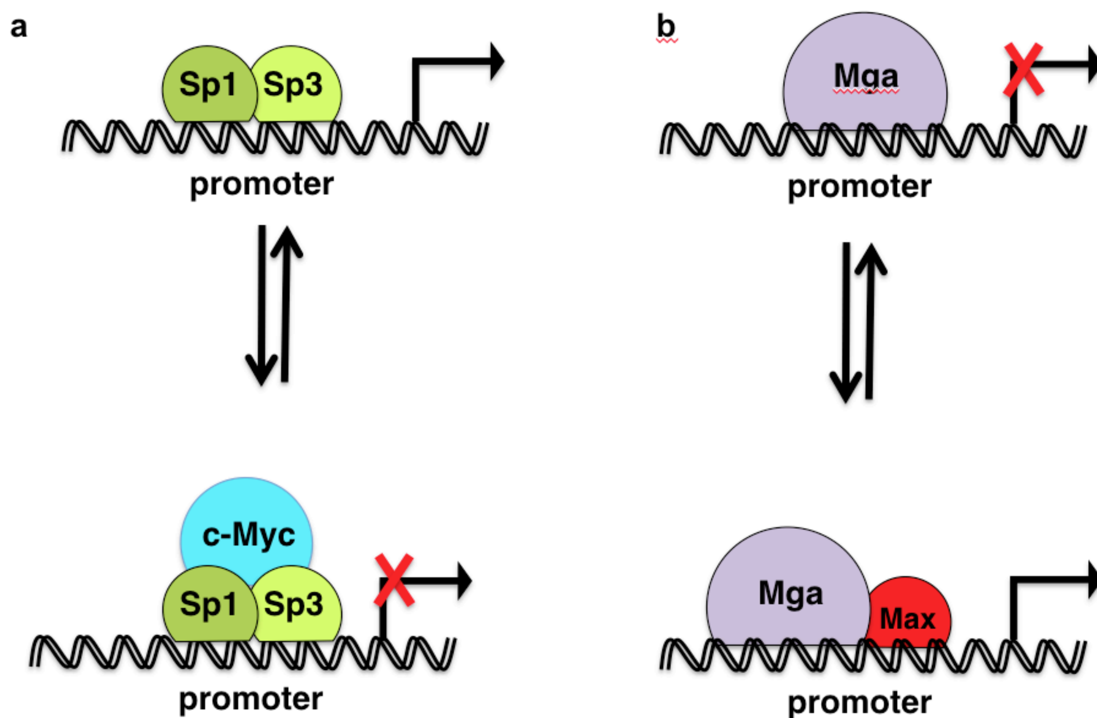


Fig 4. Inr-independent mechanisms of repression by c-Myc (a) and Mga (b).

1.7.1. Cell cycle

c-Myc expression is elevated during embryogenesis and in rapidly proliferating adult tissues, such as epithelial and progenitor cells. c-Myc levels are regulated throughout the cell cycle. Quiescent cells *in vitro* express almost undetectable levels of c-Myc, but after mitogen or serum stimulation, c-Myc levels are induced and cells enter the G_1 phase. Deregulated expression of *c-myc* characterises oncogenic activation and is a frequent hallmark of tumour-derived cells (Adams and Cory, 1985; Dalla-Favera et al., 1982; Morgenbesser and DePinho, 1994). Many studies have shown that c-Myc regulates the cell cycle through its ability to activate or repress the expression of genes involved in cell cycle progression.

c-Myc regulates the G1/S transition, and c-Myc expression is reportedly sufficient to overcome the checkpoint and to induce S phase entry in the absence of growth factors (Eilers, 1999). In addition, studies in both Rat1a and *c-myc* $^{-/-}$ mouse embryonic fibroblasts (MEF) showed that these cells have prolonged doubling times and G1 and G2 phases (de Alborán et al., 2001; Mateyak et al., 1997).

Cell cycle progression is promoted by cyclin-dependent kinases (CDK) and their cyclin regulatory subunits. Deregulated c-Myc expression leads to direct or indirect activation of the *cyclin* D_1, D_2, *E* and *A* genes (Bouchard et al., 1999; Daksis, 1994; Hoang et al., 1994), as well as *cdk4* (Hermeking et al., 2000) and *Cdc25A* (Galaktionov et al., 1996), a phosphatase that activates CDK2 and CDK4. c-Myc also suppresses cyclin kinase inhibitors such as $p15^{ink4b}$, $p21^{cip1}$ and $p27^{kip1}$ (Dang, 1999; Grandori et al., 2000).

The activity of both CDK2 and CDK4 result in retinoblastoma protein (Rb) hyperphosphorylation and release of E2F from Rb, which regulates genes implicated in replication and mitosis (Humbert et al., 2000; Ishida et al., 2001; Muller et al., 2001).

1.7.2. Apoptosis

c-Myc has an important role in apoptosis. Ectopic c-Myc expression in serum-starved fibroblasts leads to apoptosis and loss of the entire population (Evan et al., 1992); indeed, subsequent studies showed the involvement of many c-Myc target genes in the process of apoptosis (Morrish et al., 2003). c-Myc induces apoptosis by at least four different mechanisms. In the first case, it induces activation of BAX, a pro-apoptotic molecule that also induces cytochrome c release from mitochondria (Soucie et al., 2001). In a second mechanism, it induces cytochrome c release from mitochondria, which in turn associate with APAF1 protein, activating pro-caspase 9 and promoting the downstream caspase cascade, leading to apoptosis (Juin et al., 1999). A third pathway is enhancement of cell sensitivity to signalling through the CD95 death receptor, which triggers association of the intracellular adaptor protein FADD with the CD95 receptor, recruiting pro-capase 8 and leading to activation of the caspase cascade (Hueber et al., 1997). Finally, c-Myc induces apoptosis by indirect activation of p53 via ARF, which activates the p19/Mdm2/p53 apoptotic pathway (Zindy et al., 1998).

1.7.3. Cell growth

All organisms must coordinate their increase in cell size with division (Neufeld and Edgar, 1998). Although the mechanism by which c-Myc regulates cell growth is not yet well understood, there are some candidate target genes that regulate protein synthesis as well as cell metabolism, such as *eIF2a*, *eIF4E*, *RNA helicase MrDb*, *IRP-2* and H-*ferritin* (Grandori et al., 2000). c-Myc overexpression in flies causes tissue growth by increasing cell size (Johnston et al., 1999) and in mammals also produces larger cells (i.e., in B lymphocytes of Em-myc transgenic mice at different stages) (Irritani and Eisenman, 1999). After BCR- or mitogen-induced activation, primary B lymphocytes progress from small quiescent B cells to large B cell blasts (Thompson et al., 1984). Following treatment with anti-CD40 plus interleukin 4 (interleukin-4), however, c-Myc-deficient B lymphocytes are unable to increase in size (de Alborán et al., 2001). Ectopic expression of *c-myc* in liver promotes enlargement of hepatocytes in the absence of proliferation or apoptosis (Kim et al., 2000), whereas *c-myc* inactivation in liver causes a decrease in hepatocyte size, as well as

smaller cell area (Baena et al., 2005).

Recent studies show that c-Myc regulates the expression of genes transcribed by RNA polymerase III (such as *tRNA* and 5S rRNA) and RNA polymerase I (genes encoding ribosomal RNA) (Dai et al., 2010).

1.7.4. B lymphocyte differentiation

In addition to its functions in cell proliferation, apoptosis and cell growth, c-Myc plays an important role in cell differentiation. c-Myc is normally expressed in immature proliferating cells and its downregulation is reported to trigger cell differentiation (Chang et al., 2000; Henriksson and Luscher, 1996. Ectopic *c-myc* expression blocks terminal differentiation in many cell types, both *in vivo* and *in vitro* (Facchin and Facchin, 1998; Iritani and Eisenman, 1999). In B lymphocytes, Blimp-1 overexpression in a pro-monocytic cell line triggers transcriptional repression of *c-myc*, which induces differentiation to macrophages and B cells (Chang et al., 2000). Overexpression of *c-myc* in *Eμ-c-myc* mice leads to accumulation of immature pre-B cells in the bone marrow of these animals (Iritani and Eiseman, 1999).

B cell development is a highly regulated process whereby functional peripheral subsets are produced from haematopoietic stem cells, in the foetal liver before birth and in the bone marrow afterward (Osmond and Everett, 1964); it further proceeds in secondary lymphoid organs (such as spleen, Peyer's patches and lymph nodes). B cells differentiate from haematopoietic precursors and then undergo receptor rearrangement, resulting in the establishment of a diverse repertoire of B lymphocytes able to meet most of the antigenic challenges encountered in life.

Receptor diversification is a stochastic process that is largely antigen-independent, although signals delivered through the antigen receptor play an important part in regulating lymphocyte development. During B cell development, several key events take place. First, the B cell population expands from a small number of precursors into a very large population of immature B cells, which emigrate to the periphery to compete for entry in the recirculating repertoire of naïve mature B cells (Hardy and Hayakawa, 2001). Second, within each developing B cell, genes encoding the variable regions of immunoglobulin heavy-chain (IgH) and light-chain (IgL) molecules undergo rearrangement to diversify the repertoire of available receptors, resulting in

an estimated 10^{11} specificities (Davis and Bjorkman, 1988).
Third, the B cell repertoire is modified to eliminate or silence those lymphocytes that express antigen receptors (surface Ig) that bind self-antigen with high affinity. This developmental pathway has been thoroughly studied and characterised in the past years, revealing important growth factors and regulatory interactions and identifying different developmental stages based on cell surface expression proteins, which has given rise to many nomenclatures (Fig 5).

B lineage cells account for approximately 30% of nucleated cells in adult mouse bone marrow (Picker and Siegelman, 1999). Haematopoietic stem cells (HSC) differentiate to common lymphoid progenitors (CLP), which are B200^{+}, IL7Ra^{+}, c-Kit^{low} and ScaI^{low}. These CLP represent about 0.03% of bone marrow cells, and can give rise to B, T and natural killer cells (Kondo et al., 1997). B lineage-restricted cells, starting from pre-pro-B cells (Fig 5, fraction A), are characterised by expression of B220 and CD43 markers and the beginning of D_H-J_H rearrangement. These cells give rise to

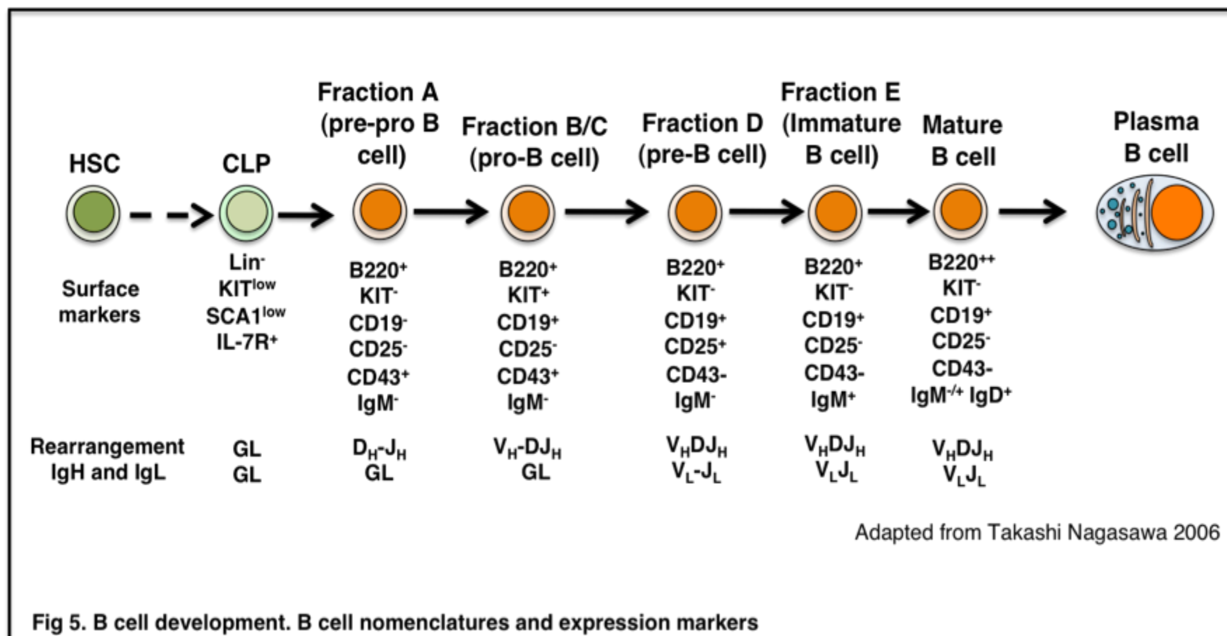


Fig 5. B cell development. B cell nomenclatures and expression markers.

pro-B cells (fraction B/C), and are characterised by expression of the CD19 surface marker and the onset of V_H-to-DJ_H rearrangement. IgH rearrangements follow an orchestrated sequence. Once productive D_H-J_H joining is complete, V_H-DJ_H

rearrangement begins. Productive V_H-DJ_H joining leads to expression of the m chain, giving rise to the pre-B cell stage, which comprises later phases (fractions C and D). The pre-B cell receptor is formed by pairing μ chain with the surrogate light chain molecules (l5 and VpreB) and the signalling molecules Igα and Igβ.

Pre-BCR expression on the surface of pre-B cells results in their increased proliferation before giving rise to the next fraction. Once a light chain gene is assembled and complete IgM receptor is expressed on the cell surface, cells are called immature B cells (fraction E). This process takes places in bone marrow and is antigen-independent, after which immature B cells move to the periphery where they are subject to selection for self-tolerance. Surviving cells undergo further differentiation to become mature B cells, characterised by IgM and IgD expression on the cell surface (naïve B cells); they recirculate through secondary lymphoid organs until they encounter their specific antigen and are activated, giving rise to plasma cells.

1.8. Transcriptional control of early B lymphocyte development

The development of mature B lymphocytes from multipotent progenitors requires the coordinated action of a number of transcription factors, such as Ikaros, PU.1, E2a, early B cell factor (Ebf1) and B cell-specific activator protein (BSAP/Pax-5). Whereas Ikaros (Georgopoulos et al., 1994) and Pu.1 (Scott et al., 1994) act in the development of multipotent progenitors, E2a (Zhuang et al., 1994), Ebf1 (Lin et al., 1995), and Pax-5 (Urbanek et al., 1994) are specifically needed for development at the time of or after commitment to the B lineage.

Several key B cell lineage genes, including *rag1*, *tdt*, l*5* and *vpreb* are regulated by Ikaros. This commitment to B lineage differentiation is signalled by initiation of rearrangement of the variable (V), diversity (D) and joining (J) segments of DNA that encode the IgH genes, and by intranuclear expression of terminal deoxynucleotide transferase (TdT), which inserts nontemplated nucleotides at V_H segment joints and requires expression of the transcription factors *e2a*, *ebf1*, *pax5* and low levels of pu.1. Expression of *e2a* and *ebf1* leads to upregulation of *Iga/b, vpreb*, *l5* and *rag1/2*, which together with *pax5* are essential for B cell identity.

1.8.1. Ikaros

The Ikaros proteins are a family of conserved zinc-finger DNA-binding transcription factors characterised by a highly conserved C2H2 zinc finger DNA-binding domain near the N terminus and a C2H2 zinc finger protein-protein interaction domain near the C terminus that mediates the formation of dimers and multimers between Ikaros family members (Georgopoulos et al., 1992; Hahm et al., 1994). Ikaros is expressed during embryogenesis only at sites of haematopoiesis, and in the adult in early B cells, T cell progenitors and mature T cells. High-affinity binding sites for Ikaros have been identified in the regulatory regions of many lymphocyte-specific genes (Molnar and Georgopoulos, 1994**)**.

The *Ikaros* gene, encoding a zinc-finger transcription factor, exerts a critical role during very early stages of B cell development. In mouse and humans, the Ikaros family has five members. Pegasus and Eos are broadly expressed, whereas Ikaros, Aiolos, and Helios are restricted to hematopoietic cells. Ikaros protein has been shown to bind to regulatory elements present in the promoter regions of *tdt* and l5 of B cells (Sabbattini et al., 2001).

1.8.2. E2a

E2a has two splice variants, E12 and E47, and is encoded by the *tcfe2a* gene. It belongs to bHLH family of transcription factors (Kadesch, 1992) and is expressed predominantly in B lineage cells. *e2a*-deficient mice show a developmental block at the pre-pro-B cell stage (Bain et al., 1994) and are unable to rearrange D_H to J_H segments due to the absence of *rag1* expression (Bain et al., 1994; Borghesi et al., 2005). *e2a* knockout progenitors show normal levels of *IL-7Ra*, *Igb* and *Igm*^{0}, but decreased *ebf1* levels and no *pax5*, *cd19*, *Iga* or *l5* expression (Bain et al., 1994; Bain et al., 1997).

The *ebf1* promoter contains E2a binding sites, and E2a activates *ebf1* expression (Roessler et al., 2007; Smith et al., 2002). Forced expression of *ebf1* in *e2a*-deficient HSC promotes development of pro-B cells *in vitro* (Bain et al., 1994; Seet et al., 2004), but these cells are unable to proliferate due to the lack of the E2a-IL-7R pathway that upregulates *N-myc* expression (Seet et al., 2004).

Studies in conditional *e2a* knockout mice showed that E2a is necessary for the

generation and development of pro-B, pre-B and immature B cells in bone marrow and for development of germinal centre B cells in the spleen, but is dispensable for generation of mature and plasma B cells in peripheral organs (Kwon et al., 2008). Gene expression analysis of these *e2a* conditional knockout pro-B cells indicated that *ebf1*, *pax5, cd19, cd79a, cd79b and Igll1* levels are downregulated compared to those of WT pro-B cells. E2a therefore controls expression of *ebf1*, acts upstream of *pax5*, and is needed for maintenance of the gene expression programme of pro-B cells.

1.8.3. Ebf1

Early B cell factor 1 (Ebf1) is a member of the COE (Collier Olf EBF) family of transcription factors that bind DNA through a zinc motif in the N-terminal domain (Hagman et al., 1993; Hagman and Lukin, 2005); it contains a C-terminal domain related to the HLH domain and mediates Ebf1 homodimer formation. Ebf1 is expressed in brain, adipose tissue, olfactory neurons (Wang and Reed, 1993) and in all B cell stages except plasma cells, where its expression is downregulated (Hagman and Lukin, 2005). This protein can bind DNA as a monomer or homodimer on correctly spaced DNA half-sites.

Expression of the *ebf1* gene involves two promoters that are differentially regulated and gives rise to two isoforms, Ebf1β and Ebf1β, which differ in the first 14 amino acids but have the same transactivation potential (Roessler et al., 2007). One distal promoter, termed *ebf1α* promoter, is activated by E2a, the IL-7 pathway and by Ebf1 itself in a feedback loop (Smith, 2002), and a proximal promoter, termed *ebf1β* promoter, is controlled by Pax5 and ETS1 proteins. *ebf1* expression is therefore tightly controlled by a well-regulated mechanism in which E2a activates *ebf1* expression by binding the *ebf1α* promoter, and Ebf1α binds and activates the *Pax5* promoter; this in turn binds the *ebf1β* promoter and regulates *ebf1β* expression in a feedback loop.

Several studies have shown a role for Ebf1 in the B cell specification program. For example, retroviral expression of *ebf1* in HSC promotes B cell differentiation (Zhang et al., 2003). Ectopic *ebf1* expression rescues B cell development in *e2a*-deficient HSC (Bain et al., 1994; Seet et al., 2004), in *pu.1* knockout HSC (Medina et al., 2004)

and in IL-7R-deficient pre-pro-B cells and CLP (Dias et al., 2005; Kikuchi et al., 2005). In all these cases, *pax5* expression does not rescue B lymphocyte development, indicating that Ebf1 might have other activities in addition to activation of the *pax5* gene.

Forced *ebf1* expression in *e2a* or *pu.1*-deficient progenitors results in the activation of *pax5* gene expression (Akerblad et al., 1999; Medina et al., 2004). Overexpression of *ebf1* and *e2a* in Ba/F3 cells nonetheless does not induce *pax5*, suggesting that *pax5* activation by *ebf1* might require additional transcription factors expressed in lymphoid progenitor cells, but not in Ba/F3 cells (Roessler et al., 2007).

1.8.4. Pax5

Pax5 is one of nine mammalian Pax transcription factors, yet it is the only Pax protein expressed in the haematopoietic system. The *pax5* gene encoding the B cell-specific activator protein (BSAP or Pax5) is the master regulator of B cell commitment. Pax5 was first identified as a DNA-binding factor that interacts with segments of the immunoglobulin heavy-chain (Igh) and klight-chain (Igk) gene loci (Adams et al., 1992). Pax5 was discovered independently as a DNA-binding protein with the same DNA sequence specificity as the sea urchin transcription factor TSAP (Barberis et al., 1989). Biochemical purification and cDNA cloning showed that BSAP is encoded by the *Pax5* gene.

pax5$^{-/-}$ pro-B cells can completely restore thymocyte development in *RAG2*-deficient mice (Rolink et al., 1999) and differentiate efficiently *in vitro* into T cells during culture with OP9 stromal cells expressing the Notch ligand Delta-like 1 (DL1). *pax5*$^{-/-}$ pro-B cells are unable to differentiate into mature B cells, however, unless their B cell potential is rescued by retroviral restoration of Pax5 expression (Nutt et al., 1999). Pax5 is therefore considered the critical B cell lineage commitment factor that restricts the developmental options of lymphoid progenitors to the B cell pathway.

1.9. Terminal B cell differentiation

The principal function of B cells is to produce antibodies against antigens. Antibody production involves three different processes: VDJ recombination, Somatic Hypermutation (SHM) and Class Switch Recombination (CSR). VDJ recombination takes place in the bone marrow, where immunoglobulin variable regions from heavy and light chains of Ig genes are rearranged to produce a primary repertoire of IgM antibodies with low affinity binding for antigen.

Once naïve mature B cells encounter an antigen, they present it through major histocompatibility complex (MHC) class II to $CD4^+$ T cells (T-helper cells). This contact causes activation of the B cells, which proliferate and migrate into the dark zone of the germinal centre (GC) in peripheral lymphoid organs such as spleen, lymph node, tonsil and Peyer's patches, where they become centroblasts.

This differentiation to centroblasts is associated with expression of activation-induced cytidine deaminase (AID), which alters the Ig genes by triggering SHM and CSR (Fig 7). When these processes are complete, those cells that have acquired higher affinity for antigen due to successful SHM are positively selected to differentiate into plasma or memory B cells, in which CSR has modified the antibody isotype, and to migrate to the light zone of the GC. Those B cells that have not undergone affinity maturation or have become autoreactive either undergo apoptosis or become anergic (MacLennan 1994).

1.9.1. Class switch recombination

CSR alters the isotype of antibody produced by a B cell from IgM or IgD to IgG, IgE or IgA, adding diverse effector functions while preserving antigen specificity. The heavy chain constant region (C_H) in mouse has eight genes, organized as Cβ Cδ, Cγ3, Cγ1, Cγ2b, Cγ2a, Cε and Cα (Fig 6).

CSR takes places between large repetitive switch regions located upstream of each C_H region gene, except for Cδ, with the excision and loss of all intervening sequences (Stavnezer et al., 2000). Once CSR occurs, the prior Cm constant region expressed during development is replaced with one of the downstream C_H exons (Cγ3, Cγ1, Cγ2β, Cγ2α, Cε or Cα). This region confers different properties on the antibody, such as the ability to bind complement, to bind to Fc receptors, to polymerize, to increase avidity, to diffuse into tissues or to remain in the circulation.

To carry out the CSR process, transcription is necessary through each of the C_H regions in which recombination has to take place. This transcription produces a sterile or germline transcript (GLT) that is spliced, but does not encode a protein (Fig 6). This GLT remains stably associated with the DNA template strand and forms

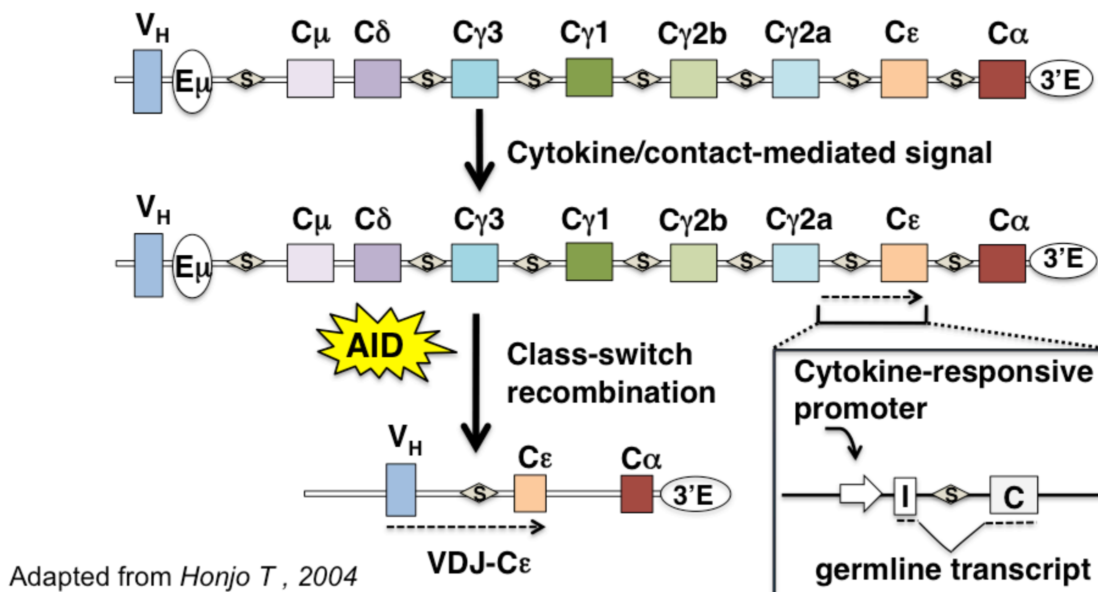


Fig 6. AID regulates CSR. The C_H region exons are switched from Cμ to one of the downstream C genes. This process depends on transcription of the target gene segment (Germline transcript-GLT) and the expression of AID, both of which are induced by cytokines and others stimuli. Upstream of the I exon is a promoter responsive to specific cytokine. The recombination target is determined by the activation of the specific germline promoter.

RNA-DNA hybrids, allowing formation of many loops in the non-template, single-stranded DNA due to the presence of G-rich sequences (R loop structure) (Shinkura et al., 2003; Yu et al., 2003). Each of the promoters of the S regions is differentially transcribed in response to different mitogens and cytokines; this mechanism directs the class switch to specific isotypes (reviewed in Stavnezer, 2000) (Fig 6). Lipopolysaccharide (LPS) induces expression of GLT and class switching to γ2b and γ3, whereas anti-CD40 plus interleukin-4 induces switching to γ1 and ε (reviewed in Coffman et al., 1993). In addition, the presence of enhancers located at the 3' end of the heavy chain locus appear to regulate the CSR, since deletion of some causes loss of switching to certain isotypes (Pinaud et al., 2001).

1.9.2. Activation-induced cytidine deaminase (AID)

In addition to GLT expression, B cells must express AID protein to carry out the CSR process. AID was originally described as a B cell-specific factor unique to activated germinal centre B cells. In this setting, AID expression is induced by signals that induce CSR in naïve B cells, such as exposure to pathogenic stimuli, T cell help, and specific cytokines. AID initiates CSR by introducing G:U mismatches into the Sm and recipient S switch regions (Fig 7) by deaminating cytidine residues in single-stranded DNA (Bransteitter et al., 2003; Chaudhuri et al., 2003; Dickerson et al., 2003; Pham et al., 2003; Ramiro et al., 2003; Sohail et al., 2003). The resulting G:U mismatches are processed by several different DNA repair mechanisms to produce mutations or DNA double-strand breaks (Di Noia and Neuberger, 2007; Peled et al., 2008), which after a regulated process, give rise to CSR. AID also contributes to malignant transformation by initiating chromosome translocations (Ramiro et al., 2004, 2006; Robbiani et al., 2008; Jankovic et al., 2010). AID is a 198-amino-acid enzyme (Muramatsu et al., 1999) that deaminates cytosine residues embedded in WRCY consensus sequences (where W = adenosine/thymine, R = purine, and Y = pyrimidine) (Rogozin and Kolchanov, 1992). This preference is dictated in part by the composition of the active site (Wang et al., 2010), although it cannot fully account for AID target choice. While several AID cofactors have been reported, including replication protein A (RPA), protein kinase-Ar1a, and CTNNBL1, none of these are known to impart specificity to AID (Basu et al., 2005; Chaudhuri et al., 2004;

Conticello et al., 2008; McBride et al., 2006; Pasqualucci et al., 2006).

1.9.3. Class switch recombination and cell division

There is a well-documented relationship between cell division and CSR, both in human and in murine B cells, showing evidence of a division-linked increase in the frequency of cell surface expression of switched Ig isotypes after stimulation (Tangye and Hodgkin PD., 2004). Division-linked CSR operates at both the molecular and cellular levels, increasing the number of switched cells with successive divisions in a time-independent manner (Rush et al., 2005). Undivided cells are unable to switch even after 4 days of *in vitro* stimulation; this is due to low AID mRNA expression, as well as to low GLT and H3 acetylation levels indicating that the switch regions are not fully accessible in undivided cells. It is also necessary that single-strand lesions generated by the combined action of AID and the mismatch repair (MMR) and base-excision repair (BER) pathways are converted into double-strand breaks by replication of the IgH locus during S phase. In addition, certain DNA repair pathways involved in CSR operate in specific cell cycle stages, which might prevent recombination until the cell enters and completes these stages. It was therefore proposed that at least one round of DNA replication is required for CSR (Rush et al., 2005; Lundgren et al., 1995).

2. AIMS

2. Aims

2.1. To define c-Myc target genes involved in early B cell differentiation using conditional mouse models.

2.2. To characterize the role of c-Myc in heavy chain class switch recombination.

3. **MATERIALS & METHODS**

3. **Materials and Methods**

3.1. Mice

To generate *c-myc*$^{fl/fl}$*;mb-1*$^{cre/+}$ mice, *c-myc*$^{fl/fl}$ were bred with *mb-1*$^{cre/+}$ mice (Hobeika et al., 2006) and progeny crossbred to yield homozygous (*c-myc*$^{fl/fl}$*;mb-1*$^{cre/+}$) and control mice (*c-myc*$^{fl/+}$*;mb-1*$^{cre/+}$). nMice were genotyped using a PCR-based analysis of tail genomic DNA (de Alborán et al., 2004; Baena et al., 2005). The primers *hcre-DIR* (5'-ACCTCTGATGAAGTCAGGAAGAAC-3'), *hcre-REV* (5'-GGAGATGTCCTTC ACTCTGATTCT-3'), *mb-1in1* (5'-CTGCGGGTAGAAGGGGGTC-3') *and mb-1in2 (*5'-CCTTGCGAGGTCAGGGAGCC-3') were used to amplify *mb-1-cre* (hcre-DIR and hcre-REV) and mb-1-wt alleles (mb-1in1 and mb-1in2).

The *rosa26-egfp* allele was genotyped as described (Mao et al., 2001). To generate *c-myc*$^{fl/fl}$ *cd19*$^{cre/+}$ mice first we bred *c-myc*$^{fl/fl}$ *with cd19*$^{cre/+}$ (Rickert et al., 1997) and progeny bred to yield homozygous *(c-myc*$^{fl/fl}$*cd19*$^{cre/+}$) nd control mice *(c-myc*$^{fl/+}$*cd19*$^{cre/+}$*).* Briefly, *c-myc*$^{fl/fl}$*cd19*$^{cre/+}$ were bred with *rosa26*$^{egfp/egfp}$ mice (Mao et al., 2001) and progeny crossbred to yield homozygous *c-myc*$^{fl/fl}$*cd19*$^{cre/+}$*rosa26*$^{egfp/egfp}$ or heterozygous *c-myc*$^{fl/+}$*cd19*$^{cre/+}$*rosa26*$^{egfp/egfp}$ *mice.* To generate *c-myc*$^{fl/fl}$*cd19*$^{cre/+}$*rosa26*$^{egfp/egfp}$ *IgK-AID,* we crossed *c-myc*$^{fl/fl}$*cd19*$^{cre/+}$*rosa26*$^{egfp/egfp}$ with *Igk-AID* transgenic mouse (Robbiani et al,. 2009).

Mice were genotyped using a PCR-based analysis of tail genomic DNA (de Alborán et al., 2004; Baena et al., 2005). To amplify the different alleles, we used the following primer pairs: floxed *c-myc* allele, *Flox S* (5'-GCCCCTGAATTGCTAGGAAG ACTG-3') and *Flox A* (5'-CCGACCGGGTCCGAGTCCCTATT-3') (530 bp product). *cd19-cre* allele*, Cre 3* (5'-CAATTTACTGACCGTACA-3') and *Cre 4* (5'-CATCGCCAT CTTCCAGCA-3') (1000 bp product). *Cd19 wt* allele, *Cd19.8* (5'-AATGTTGTGCTGC CATGCCTC-3') and *Cd19.9* (5'-GTCTGAAGCATTCCACCGGAA-3') (550 bp product). *Rosa26-egfp* allele, *lac-Z1* (5'-GTGGTGGTTATGCCGATCG-3') and *lac-Z2* (5'-TACCACAGCGGATGGTTCGG-3'). For the AID tg allele, oIMR7486 (5'- GTGGC TGAGTTTCTGAGATGGAAC-3') and oIMR7487 (5'- AACAGTGGTAGGTCGCTT GTGG-3') (434 bp product). *aicda* KO mice were crossbred to yield homozygous knockout mice and were genotyped as described (Muramatsu et al, 2000), using the primers AID13, AID14, AID15.

3.2. Flow cytometry analysis and cell sorting

For cell sorting or flow cytometry analysis, bone marrow B lymphocytes were purified (FACS Aria cell sorter) as $Ly6c^-NK1.1^-DX5^-B220^+$ $c\text{-}Kit^+$ (pro-B) and $Ly6c^-NK1.1^-DX5^-$ $B220^+CD25^+$ (pre-B) cells. Purity >97% was verified by flow cytometry re-analysis. Anti-B220 antibodies were conjugated either with PeCy7 (Bioscience), FITC or APC (Becton Coulter). Anti-IgM antibodies (Southern Biotechnologies) were conjugated with PE or biotin. APC anti-CD19 antibody was from Becton Coulter. PE-anti-CD25, biotin-anti-CD43, PE-anti-CD117, and biotin-anti-pre-BCR antibodies were all from Pharmingen. APC-streptavidin (Pharmingen) or ECD-streptavidin (Immunotech) was used to conjugate with biotin. FITC- or biotin-conjugated anti-Ly6.C (Becton Coulter), -NK1.1 and -DX5 (both from Pharmingen) antibodies were used in a dump channel to remove contaminating NK and dendritic cells. Antibodies for FACS were from Pharmingen (IgG1-PE, IgE-PE, IgG2b-biotin, IgG3-PE, streptavidin-PE).

3.3. Gene expression analysis

For qPCR analysis, 2.5 μl cDNA (ten-fold dilution series) was mixed with primers and SYBR Green PCR master mix (Becton Dickinson). All oligonucleotides were designed to yield 70-130 bp PCR fragments. Oligonucleotides for *c-myc* and *β-actin* were as described (Campanero et al., 2000). Primers for *cd19* were *cd19 sense* (s) (5'-AGTACGGGAATGTGCTCTCC-3') and *cd19 antisense* (as) (5'-GGACTTGAATGC GTGGATTT-3'), *E2a-s* (5'-ATACAGCGAAGGTGCCCACT-3') and *E2a-as* (5'-CTCAA GGTGCCAACACTGGT-3') for *tcfe2a*, *Ebf1-s* (5'-CTATGTGCGCCTCATCGACT-3') and *Ebf1-as* (5'-CATGATCTCGTGTGTGAGCAA-3') for *ebf1*, *Flt3-s* (5'-CAGCCGCA CTTTGATTTACA-3') and *Flt3-as* (5'-GGCTTCGCTCTGAATATGGA-3') for *flt3*. Each gene was analysed in triplicate. cDNA samples and reagents were run on an ABI Prism 7900HT. Data were analysed with SDS 2.2 sequence detection systems.

3.4. Luciferase activity assay

The murine *ebf1 α*, *ebf1 β*, *e2a* and *pax5* promoters were amplified by PCR using the following primers: *ebf1-α-FWD*: 5'-TAAGAGCGCGGAACTGTCC-3', *ebf1-α-REV:* 5'-GCTGAAGAATCTGCCAGAAGTT-3', *ebf1β-FWD* 5'-CTAGAATGCCCATTCC CTTG-3', *ebf1β-REV* 5'-CCTTTTCTTGTGGAAAAATC-3', *e2a-FWD* 5'-

CCGCTCGAA CACATAACACC -3', *e2a-REV* 5'-GGAAGGTTGGAAGTTCTGAGG-3', *pax5-FWD* 5'-TGCGGGCACTCGGAGCCGAA-3', *pax5-REV* 5'-GATACTAGCACTGGGTAGTG-3', respectively, cloned into the EcoRI site of the Topo 2.1 vector (Invitrogen) and subcloned into the NheI-BglII site of the pGL3-Control vector (Promega) upstream of the luciferase gene to generate *pGL3-ebf1*α, *pGL3-ebf1*β, *pGL3-e2a* and *pGL3-pax5* vectors.

For luciferase assays, HEK-293T cells were plated in 24-well plates at 2×10^5 cells/well, cultured to subconfluence, and cotransfected using JetPeI reagent (PolyPlus) with 500 ng *pGL3-ebf1*α, *pGL3-ebf1*β, *pGL3-e2a* and *pGL3-pax5* or *pGL3* control vector and increasing amounts of *pRV-IRES-gfp-c-myc* expression vector. At 24 h post-transfection, firefly and renilla activity were measured using the Dual-Luciferase Reporter Assay (Promega).

L1-2 cells (5×10^6) were transfected by electroporation using 5 μg of *pGL3-ebf1*α, *pGL3-ebf1*β, *pGL3-e2a* and *pGL3-pax5* or *pGL3* control vector and increasing amounts of *pRV-IRES-gfp-c-myc* expression vector. At 18 h post-transfection, firefly and renilla activity were measured using the Dual-Luciferase Reporter Assay.

Murine *aicda* regions were amplified by PCR using the following primers: *R1*.Fwd, 5'-GAAGATCTTGTGTCTCAGTATGTCATTCC-3', *R1.Rev*, 5'-ATGCCATGGGGAGCAC ATGCACAAGCAGAT-3'; *R4.*Fwd, 5'-GACGCGTGGAACTCTCTGTAACCTC GGGC-3', *R4.Rev, 5'-CGACGCGTGCAAGGCAGCGAGGACAGAG*-3'; *I1.Fwd*, 5'-CGCGGATCCGACAGTGGAGAGACACAG-3', *I1.Rev*, 5'- CCGCTCGAGGGTAAGG AGGACTTTG CTAG-3' cloned into the BglII-NcoI, MluI-NheI and BamHI-XhoI sites, respectively, of the *pGL3-empty* vector (Promega), up- or downstream of the luciferase gene to generate vectors *pGL3-R1, pGL3-R1-I1, pGL3-R1-R4, pGL3-R1-R4-I1*. For luciferase assays, HEK 293T cells were plated in 24-well plates at 2×10^5 cells/well, cultured to subconfluence, and cotransfected using JetPeI with 500 ng of *pGL3-R1, pGL3-R1-I1, pGL3-R1-R4 or pGL3-R1-R4-I1* and increasing amounts of *pRV-IRES-gfp-c-myc* expression vector. At 24 h post-transfection, firefly and renilla activity were measured using the Dual-Luciferase Reporter Assay. M12 cells were cotransfected by electroporation using 10 μg of *pGL3-R1, pGL3-R1-I1, pGL3-R1-R4*

or *pGL3-R1-R4-I1* and increasing amounts of *pRV-IRES-gfp-c-myc* expression vector. At 18 h post-transfection, firefly and renilla activity were measured as above. Results were represented as relative luciferase units or x-fold activation compared with empty vector.

3.5. ChIP assays

Experiments were performed following the protocol of the ChIP assay kit (Active Motif). Briefly, L1-2 and M12 B cell lines were crosslinked with formaldehyde (1% final concentration), and incubated (room temperature, 20 min). After sonication, rabbit polyclonal anti-c-Myc N262 antibody (sc-764, Santa Cruz) or pre-immune serum was used to precipitate chromatin from 2 x 10^6 cells. Immunoprecipitated DNA and input samples were analysed with SYBR Green RT-PCR Kit (Applied Biosystems) and the percentage of enrichment relative to the amount of input chromatin was determined as $2(Ct^{input} - Ct^{antibody})$. Primers flanking E-box5 in the *ebf1* α promoter were: *E5-FW* (5'-CCTCAGCTCGTTCTGAGAGG-3') and *EB5-REV* (5'-ACTCGCAGGAGGTAGAG AACG-3).

3.6. Electrophoretic mobility shift assays (EMSA)

EMSA was performed with labelled double-stranded (ds) oligonucleotides encompassing E-boxes from the *ebf1* α and *dhfr* promoters. pcDNA3-c-Myc and pcDNA3-Max were *in vitro*-translated (IVT) using TNT-coupled reticulocyte lysate systems (Promega). Binding reactions between IVT proteins and labelled probes (1 ng) were performed as described (Kuhn, et al. 1995), except that 0.25x TBE was used. Unlabelled oligonucleotide competitors (100 ng) and either 1 mg anti-c-Myc sc-764 or 1 mg anti-c-Myb sc-517 antibody (both from Santa Cruz) were used. Ds oligonucleotides used: *dhfr-I-wt* (5'-GGCGCGACACCCACGTGCCCT-3' and 5'-AGAGAGGGCACGTGGGTGTCG-3'), *EB5-wt* (5'-GGTCCTACCCACGTTGACTG CAGT-3' and 5'GAGACTGCAGTCAACGTGGGTAGGA-3'), *EB5-mut* (5'-GGTCCTAC CCTTGCTGACTGCAGT-3' and 5'-GAGACTGCAGTCAGCAAGGGTAGGA-3'), *EB505-wt* (5'-CGTTTCCTCACCTGTACAATGGGAGTGG-3' and 5'-GTCCACTCCCA TTGTACAGGTGAGGAAA-3'), *EB505-mut* (5'-CGTTTCCTCTCTTATACAATGGGA GTGG-3' and 5'-GTCCACTCCCATTGTATAAGAGAGGAAA-3'). Complementary

oligonucleotides were mixed at an equimolar ratio in 10 mM Tris (pH 7.5)-50 mM NaCl, heated to 65°C, and annealed by slow cooling to room temperature. Double-stranded oligonucleotides (100 ng) were labelled by a Klenow fill-in reaction.

3.7. Spleen B cell culture

Homogenised single-cell suspensions from *c-myc*$^{fl/+}$;*cd19*$^{cre/+}$*rosa26*$^{egfp/egfp}$, *c-myc*$^{fl/fl}$*cd19*$^{cre/+}$*rosa26*$^{egfp/egfp}$ and *c-myc*$^{fl/fl}$;*cd19*$^{cre/+}$;*rosa26*$^{egfp/egfp}$;*IgK-AID* were depleted of red blood cells by hypotonic lysis and stained with Pecy7-conjugated anti-B220 monoclonal antibody (Pharmingen). B lymphocytes were then sorted by flow cytometry (Coulter) based on dual cell surface expression of B220 and GFP. After sorting, purity was monitored by FACS and was usually >98%. Sorted B lymphocytes were cultured in RPMI medium supplemented with 15% foetal bovine serum, 1 mM L-glutamine, 1 mM penicillin/streptomycin, 50 μM β-mercaptoethanol and stimulated with lipopolysaccharide (LPS; 5 μg/ml) or anti-CD40 (10 μg/mL, Pharmingen) plus interleukin-4 (20 ng/mL, R&D) for 4 days at 37ºC. Cells were collected on day 4 for surface Ig analysis by FACS, after staining with PE-anti-mouse IgG1, IgG2b, IgG3 (Bioscience) and PECY7-anti-mouse CD45R (B220; clone RA3-6B2) rat mAb (BD Biosciences).

3.8. ELISA assays

Specific ELISA in 96-well-plates coated with polyclonal goat Ab F(ab)2 against the respective mouse isotype was used to measure Igγ1, Igγ2b, Igγ3, and Igε in culture supernatants of *in vitro*-stimulated spleen *c-myc*$^{fl/+}$;*cd19*$^{cre/+}$*rosa26*$^{egfp/egfp}$, *c-myc*$^{fl/fl}$*cd19*$^{cre/}$*rosa26*$^{egfp/egfp}$ and *c-myc*$^{fl/fl}$;*cd19*$^{cre/+}$;*rosa26*$^{egfp/egfp}$;*IgK-AID* B cells. Serial two-fold dilutions (1:3 to 1:9) of supernatants were added (50 μl/well) to the plates and incubated (1 h, 25°C). After washing, biotin-labelled isotype-specific mAb were added and developed using horseradish peroxidase-streptavidin. The concentration of the Ig isotypes was determined by interpolation using a calibrated standard curve for each isotype. Assays were performed in triplicates

4. **RESULTS**

4. **Results I**

4.1. *c-myc* conditional knockout

As *c-myc* inactivation in the germ line is lethal at embryonic day 9.5-10.5, a conditional knockout mouse was developed in which *c-myc* inactivation is restricted to a specific cell type. We used a *c-myc*$^{fl/fl}$ mouse with two *loxP* sites inserted between exons 2 and 3 in the mouse germ line *c-myc* locus (Fig. 7). Mice homozygous for this mutation show no phenotype in the absence of Cre recombinase (de Alborán et al., 2001). The *c-myc* floxed sequence is deleted by the expression of Cre recombinase, which triggers inactivation of the *c-myc* gene (de Alborán et al., 2001).

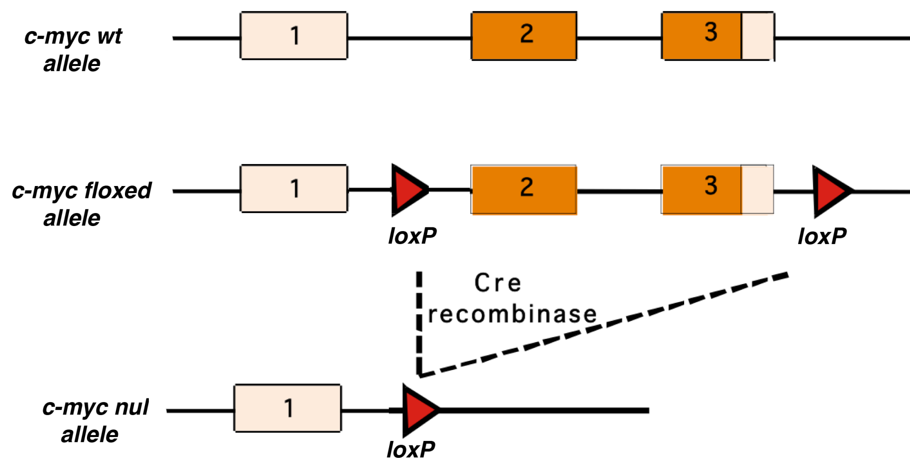


Figure 7. Targeting of the *c-myc* allele. Targeting construct containing exons 2 and 3 flanked by *loxP* sites (red triangles). When Cre recombinase is expressed, directed by a specific promoter, the region between the *loxP* sites is excised. Light-colored boxes are non-coding exons.

To study the role of c-Myc in early B cell differentiation, we conditionally inactivated the *c-myc* gene in developing B lymphocytes by breeding *c-myc*$^{fl/fl}$ mice with mb-*1*$^{cre/+}$ knock-in mice (Hobeika et al., 2006), in which Cre recombinase expression is controlled by the endogenous promoter of the *Ig*α chain (*mb-1*) gene from the earliest stage of B lymphocyte differentiation (pro- to pre-B cells) (Hobeika et al., 2006), leading to *c-myc* deletion in these B cell populations.

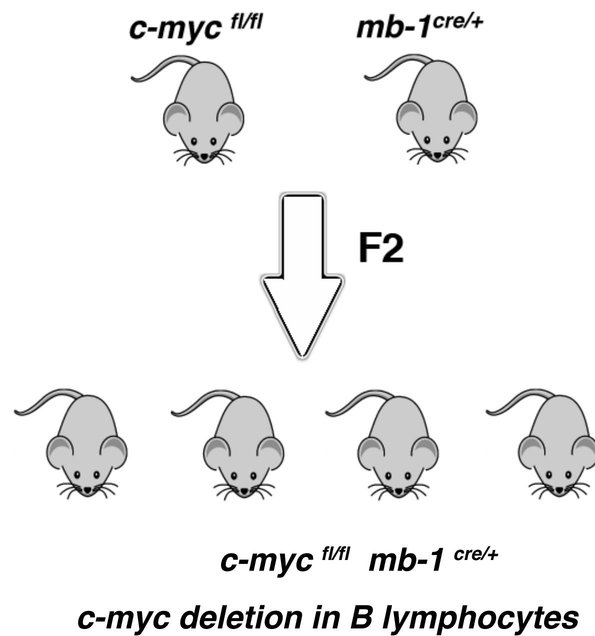
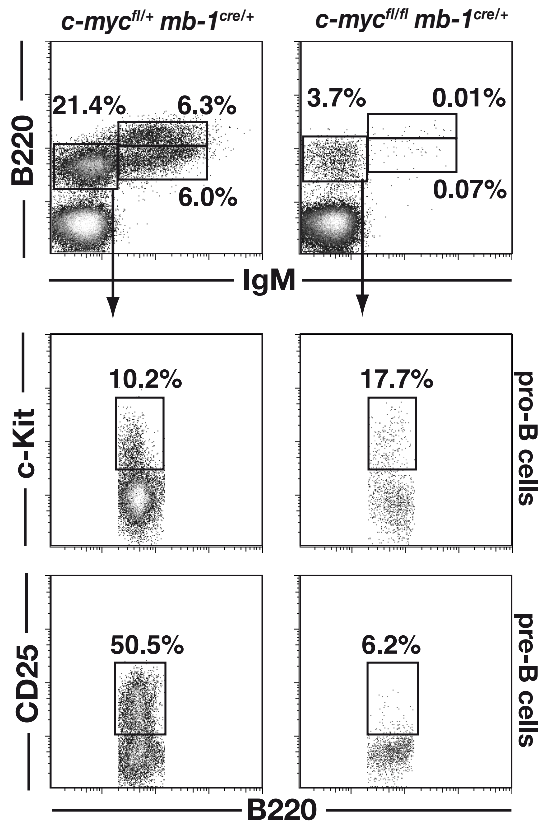


Fig 8. Scheme of the *c-myc*$^{fl/fl}$ *mb-1*$^{cre/+}$ mouse model

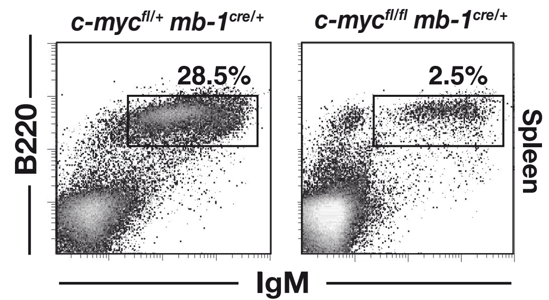
4.2. c-Myc is necessary for B lymphocyte differentiation

Previous work in our laboratory using flow cytometry to analyse B cell populations from BM and spleen (Hardy et al., 1991) of *c-myc*$^{fl/fl}$ *mb-1*$^{cre/+}$ mice showed that *c-myc* inactivation affects B cell differentiation. Deletion of the *c-myc* gene at early differentiation stages led to a developmental defect at the pro- and pre-B cell transition to the immature B cell stage (Fig. 9A) that affected the mature population in the spleen (B220^{+}IgM^{+} B cells) (Fig 9B). In addition, the absolute number of B lymphocytes (B220^{+}) in *c-myc*$^{fl/fl}$ *mb-1*$^{cre/+}$ mouse BM was four-fold lower than in controls (0.4 x 10^{6} vs 1.8 x 10^{6}) (Fig. 9C). *c-myc*$^{fl/fl}$ *mb-1*$^{cre/+}$ mouse spleens showed a 34-fold decrease (0.24 x 10^{6} vs 8.1 x 10^{6}) in the number of mature B lymphocytes compared to controls (Fig. 9C). These results indicated that c-Myc is necessary for the generation of pro- and pre-B cells.

A)



B)



C)

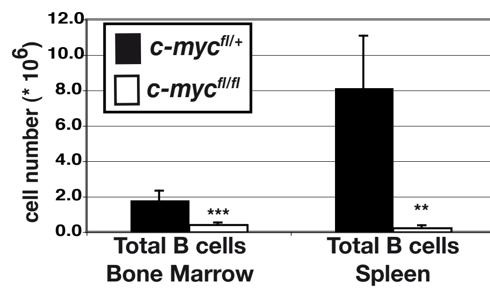


Figure 9. c-Myc is necessary for B cell differentiation. A) Single-cell suspensions of *c-myc*$^{f/+}$;*mb-1*$^{cre/+}$ and *c-myc*$^{fl/fl}$;*mb-1*$^{cre/+}$ BM were stained with anti-LyC6, Dx5, B220, IgM, c-Kit and CD25 to identify populations at different B cell differentiation stages. B220^{+}IgM^{-}c-Kit^{+} (pro-B cells) and B220^{+}IgM^{-}CD25^{+} (pre-B cells) were gated on LyC6- and DX5^{-} negative cells to remove B220^{+} non-B cells from BM. B) Single-cell suspensions of *c-myc*$^{fl/fl}$;*mb-1*$^{cre/+}$ and *c-myc*$^{f/+}$;*mb-1*$^{cre/+}$ spleen were stained with anti-B220 and -IgM to define the mature B cell population in spleen. C) Total B cells from *c-myc* $^{f/+}$;*mb-1*$^{cre/+}$ and *c-myc* $^{fl/fl}$;*mb-1* $^{cre/+}$ mouse BM and spleen.

4.3. Downregulated expression of *tcfe2a*, *efb1* and *pax5* expression in c-Myc-deficient B cells

To define the molecular mechanism by which c-Myc acts on B lymphocyte differentiation, we analysed sorted pro- and pre-B cells for the expression of key transcription factors involved in this process. Quantitative PCR (qPCR) showed that *e2a, ebf1, and pax-5* expression was slightly downregulated in sorted c-Myc-deficient pro-B cells, and that this effect was more pronounced in pre-B cells, probably due to the timing of *c-myc* deletion in *mb-1*$^{cre/+}$ mice (Fig. 4A). *e2a*$^{-/-}$, *ebf1*$^{-/-}$ and *pax-5*$^{-/-}$ mice have a block at early stages of B cell development; Ebf1 shares some target genes with Pax-5 and transcriptionally regulates its expression, providing B cell identity (O'Riordan and Grosschedl, 1999). Expression of the repressed Pax5 target gene *flt3* (Hardy et al., 2007) was increased in pro- and pre-B cells from *c-myc*$^{fl/fl}$*mb-1*$^{cre/+}$ mice compared to controls (Fig. 10).

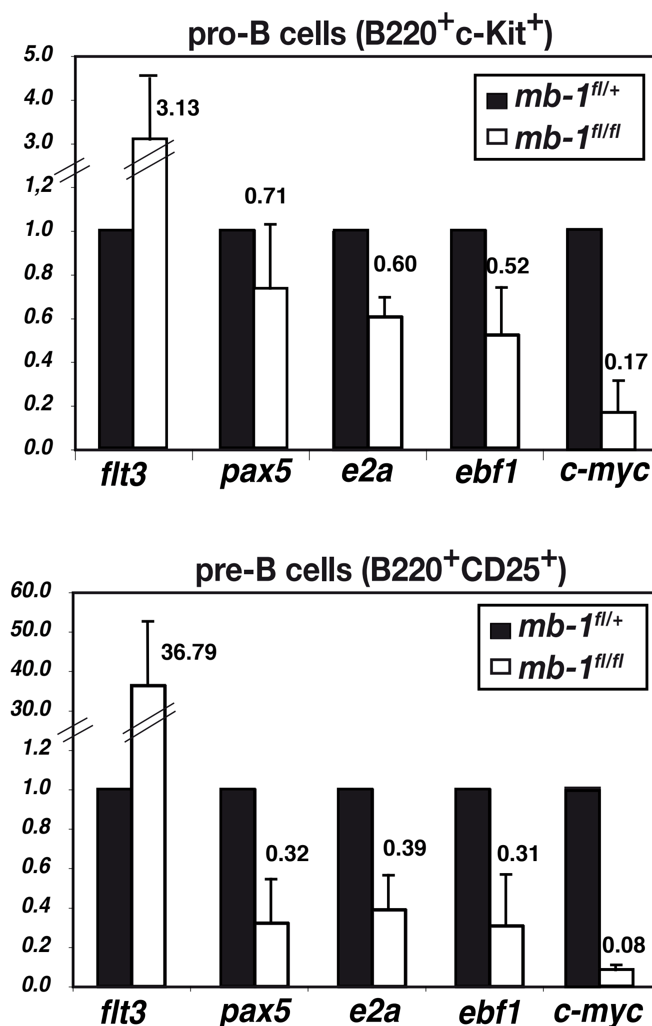


Fig. 10. Gene expression Gene expression analysis by qPCR of sorted pro- ($Ly6C^{-}NK1.1^{-}B220^{+}c\text{-}Kit^{+}$) and pre-B cells ($Ly6C^{-}NK1.1^{-}B220^{+}CD25^{+}$) from $c\text{-}myc^{fl/fl};mb\text{-}1^{cre/+}$ and $c\text{-}myc^{fl/+};mb\text{-}1^{cre/+}$ mouse BM (mean ± SD for 3 mutant and 3 control mice); numbers indicate *x*-fold change (2^(-ΔCt)).

4.4. Promoter alignment screening for c-Myc binding sites

To further study the role of c-Myc in the regulation of B lymphocyte development by controlling expression of genes important for B cell differentiation, we analysed the promoter regions and the regions 10 kb up- and downstream of the transcription start site of downregulated genes described above. We used computer alignment analysis of human and mouse genomic DNA sequences to search for conserved c-Myc-binding sites (E-boxes), we identified in *e2a*, *ebf1α*, *ebf1β* and *pax5* promoter and/or first exonic and intronic regions (Fig. 11). We found, three E-boxes in *e2a*, two in *ebf1α,* three in *ebf1β* and four in *pax5* promoters.

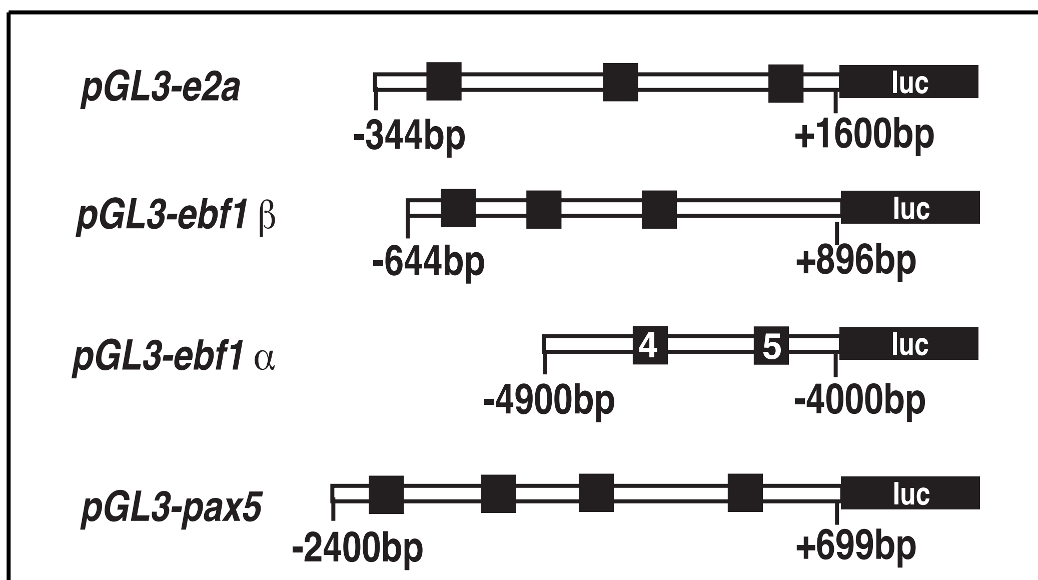


Fig 11. Scheme showing reporter constructs used in luciferase assays. E-boxes are shown as black boxes; numbers indicate distance from transcription start site.

4.5. Luciferase assays

To test whether c-Myc regulates the expression of the genes shown above, we cloned the promoter and regulatory regions into the pGL3-luc reporter vector, cotransfected them into the 293T HEK fibroblast cell line with different amounts of *c-myc* expression vector, and measured luciferase activity. *e2a*, *pax5* or *ebf1β*-pGL3-luc constructs did not activate the luciferase gene in a c-Myc dose-dependent manner (Fig. 12)).

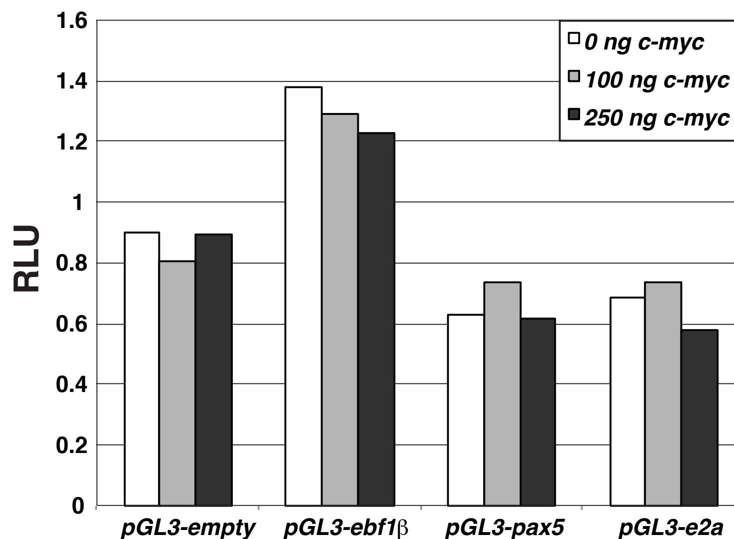
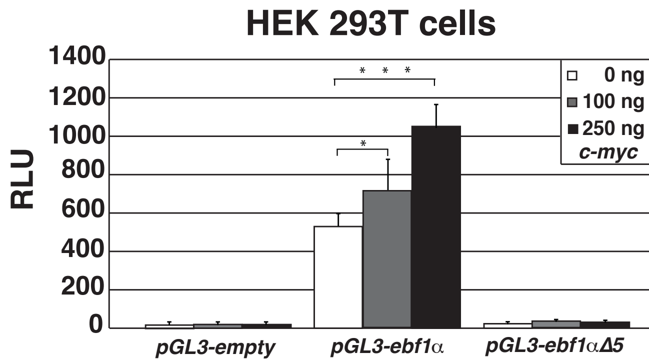


Fig 12. *ebf1β, e2a* and *pax-5* genomic loci do not respond to c-Myc. The indicated luciferase reporter constructs were cotransfected with different amounts of *c-myc* expression vector. Luciferase activity was normalised with *Renilla* activity (relative luciferase units, RLU). Figure representative of three independent experiments.

Unlike the other three constructs, the pGL3-*ebf1α* construct showed induction of luciferase activity in a c-Myc dose-dependent manner, in both HEK 293T and L1-2 lymphoid cell line (Fig 13A, B). To determine which of the two E-boxes found in the *ebf1α* promoter was responsible for this activation, site-directed mutagenesis was performed in E-box5 (EBΔ5), located 200 bp upstream of the transcription start site. Luciferase assays showed complete abolishment of basal promoter activity and c-Myc-dependent *ebf1α* transactivation in both cell lines (Fig. 13 A, B). We conclude that c-Myc activates the *ebf1α* promoter and that E-box5 is implicated in this activation.

A) B)



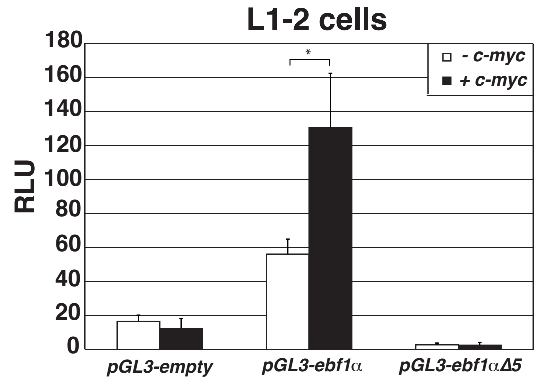


Fig 13. Luciferase assays. c-Myc transcriptionally regulates *ebf1α*. c-Myc dose-dependent activation of *ebf1α* in transient transfection assays in HEK 293T (A) and in L1-2 B cell lines (B). Luciferase activity was normalised with *Renilla* activity (RLU). Mean ± SD for three replicates in one representative experiment of 6 performed. ***p<0.001 and *p<0.05.

4.6. c-Myc binds to a region within the *ebf1α* promoter

To determine whether c-Myc binds to *ebf1α* promoter, we performed a chromatin immunoprecipitation (ChIP) assay in the L1-2 lymphoid cell line. Immunoprecipitated DNA was amplified using specific primers that flank E-box5. Using PCR, we observed 10-fold enrichment in the DNA fragments immunoprecipitated with a c-Myc-specific antibody compared to pre-immune serum (Fig 14). This result indicated that c-Myc binds to a genomic region within *ebf1α*, containing E-box5.

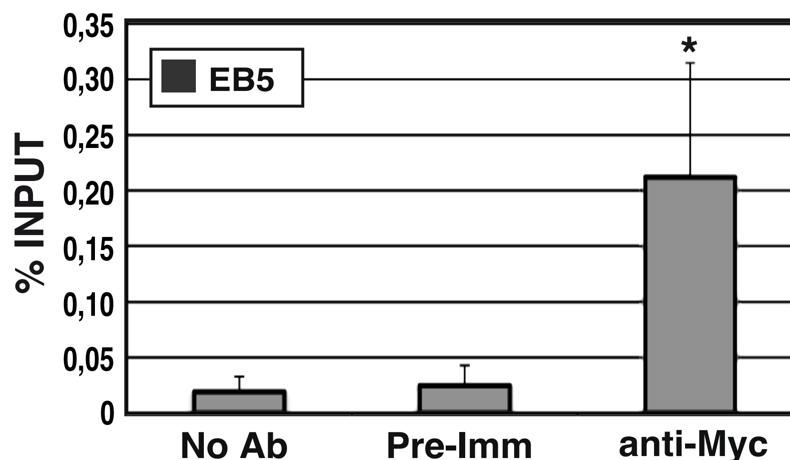


Fig 14. ChIP assays. L1-2 B cells were cross-linked and DNA was sonicated. Immunoprecipitation was performed with anti-c-Myc antibody (N262) or pre-immune serum (control). Data show the mean of three independent experiments. *$p<0.05$.

4.7. c-Myc binds to the *ebf1α* promoter through E-box5

To determine whether c-Myc bound specifically to E-box5, we perfomed EMSA assays. c-Myc bound to oligonucleotides containing E-box5. Mutated E-box5 or E-box4 did not compete for c-Myc binding with unmutated E-box5, as determined using anti-c-Myc antibody (Fig.15). As positive control for c-Myc binding to an E-box, we used the *dhfr* probe, located in a region 5´ of the *dhfr* gene (Mai et al., 1994). We observed that E-box5 from *ebf1* competed for c-Myc binding with oligonucleotides containing the *dhfr* E-box. Mutated *ebf1* E-box5 or E-box4 did not compete with the *dhfr* E-box. These data show that c-Myc directly regulates *ebf1* transcription by binding to the E-box5 in the *ebf1*α promoter.

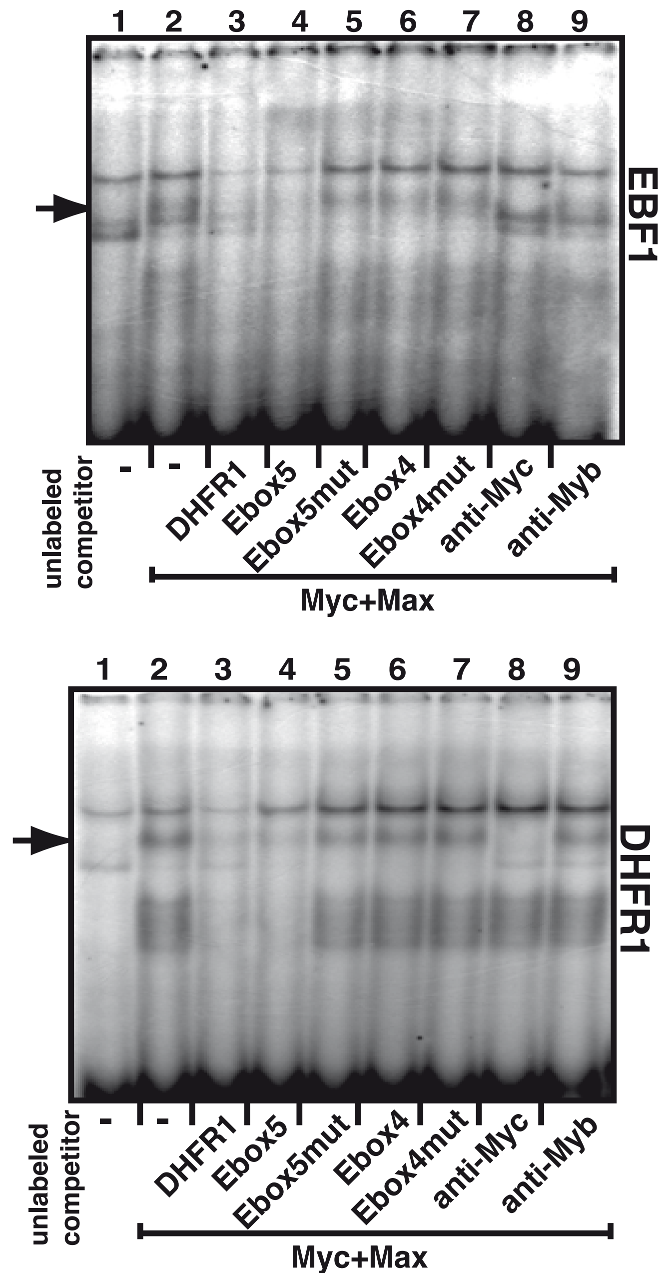


Fig 15. EMSA assay. *In vitro*-translated c-Myc and Max proteins were incubated with oligonucleotides containing E-box5 from *ebf1* (top) or the E-box from *dhfr* (bottom) and competitor oligonucleotides (indicated). Lane 1, negative control without c-Myc/Max. Lane 2, c-Myc/Max with no competitor. Lanes 3 to 7, c-Myc/Max with different competitors. Lane 8, c-Myc/Max with anti-c-Myc antibody. Lane 9, c-Myc/Max with anti-c-Myb antibody (negative control). Arrow indicates the shift of c-Myc/Max oligonucleotide complexes. All experiments are representative of at least three independent assays.

These data show that c-Myc directly regulates *ebf1* transcription by binding to E-box5 in the *ebf1*α promoter.

5. **Results II**

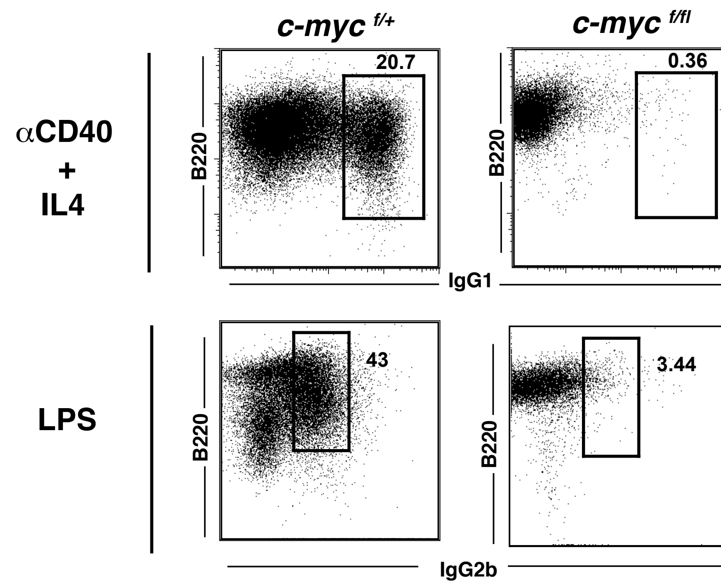
5.1. New Role of c-Myc in terminal B cell differentiation

It addition to this new role described for c-Myc in early B cell differentiation c-Myc has a critical function in terminal B cell differentiation (Shaffer et al., 2000; Lin et al., 2000), although the mechanism by which it acts is unknown. To address this question, we used the conditional *c-myc*$^{fl/fl}$*cd19*$^{cre/+}$ mouse model, in which the endogenous promoter of the *cd19* gene regulates expression of Cre recombinase (Rickert et al.,1997). To distinguish between c-myc deleted and non-deleted B cells, we crossed *c-myc*$^{fl/fl}$*cd19*$^{cre/+}$ mice with *rosa26*$^{egfp/egfp}$ reporter mice, in which green fluorescent protein (GFP) cDNA was inserted inside the *rosa26* allele (Mao et al., 2001). Expression of Cre recombinase leads to deletion of the *c-myc* allele and of the stop codon inserted in the *rosa26*^{egfp} gene, triggering GFP expression in c-Myc-deficient B lymphocytes.

5.2. c-Myc-deficient mature B lymphocytes do not undergo class switch recombination *in vitro*

To study the role of c-Myc in terminal B cell differentiation, we isolated mature ($B220^+GFP^+$) spleen B cells by sorting from *c-myc*$^{fl/+}$*cd19*$^{cre/+}$*rosa26*$^{egfp/egfp}$ and *c-myc*$^{fl/fl}$*cd19*$^{cre/+}$*rosa26*$^{egfp/egfp}$ mice (*c-myc*$^{fl/+}$ and *c-myc*$^{fl/fl}$ hereafter), and cultured them with anti-CD40 plus interleukin-4 (IL-4) or lipopolysaccharide (LPS) for 4 days. After flow cytometry analysis, we found that c-Myc-deficient B lymphocytes have a severe impairment in the generation of $IgG1^+$ cells (0.36% vs. 20.7% in *c-myc*$^{fl/+}$*)* and $IgG2b^+$ cells (3.44% vs. 43% in *c-myc*$^{fl/+}$, control mouse) (Fig 16A). In addition, we performed ELISA to test for IgG1 in *c-myc*$^{fl/+}$ and *c-myc*$^{fl/fl}$ culture cell supernatants (Fig 16B). We conclude that c-Myc-deficient B cells do not generate $IgG1^+$ or $IgG2b^+$ cells after *in vitro* activation.

A)



B)

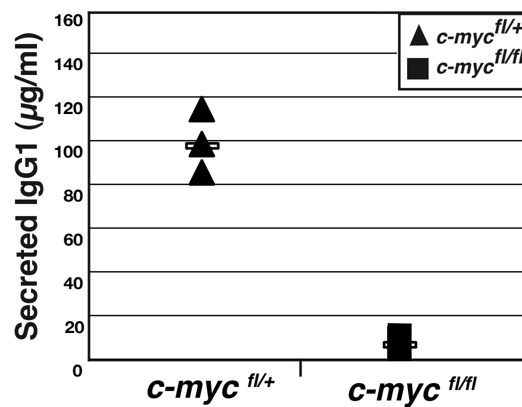


Fig 16. Impaired class switch recombination in *c-myc*-deficient activated B cells. A) Sorted B220$^+$GFP$^+$ spleen B cells from *c-myc*$^{fl/fl}$ and *c-myc*$^{fl/+}$ mice were activated with anti-CD40 plus IL-4 or LPS and analysed by flow cytometry four days later. B) ELISA for IgG1 detection in supernatants from cultures of activated B cells in A). Representative data from one of at least three independent experiments.

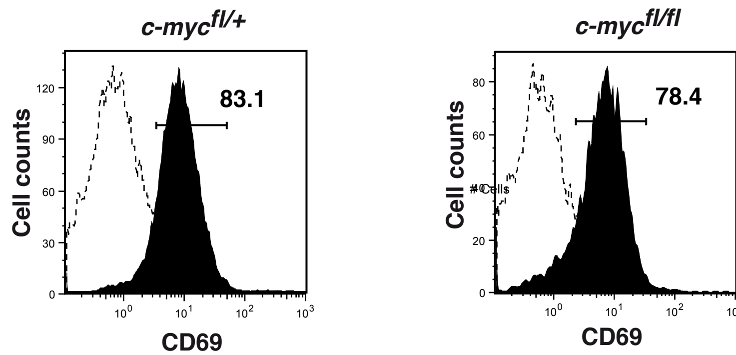


Fig 17. Normal expression of CD69 in c-Myc-deficient B cells. B220^{+}GFP^{+} mature B cells from *c-myc*$^{fl/fl}$ and *c-myc*$^{fl/+}$ mice, activated with anti-CD40 plus IL-4 and analysed for CD69 expression at day 4. Dashes lines indicate isotype control.

We tested the expression of the activation marker CD69 and observed that activated c-Myc-deficient B cells express normal levels, confirming previous findings (de Alborán et al., 2001).

5.3. Normal expression of Iγ1-Cγ1 germline transcript in activated c-Myc-deficient B lymphocytes

Class switch recombination of immunoglobulin heavy chain is regulated by cytokine stimulation, which induces transcription from an intronic (I) promoter of a specific switch (S) region, giving rise to so-called germline transcripts (GLT) (Stavnezer et al., 1986; Alt et al., 1988; Berton et al., 1989; Rothman et al., 1990; Lee et al., 2001).
It was proposed that the function of GLT transcripts is to promote S region accessibility through cotranscriptional generation of R loops and possibly other structures that provide the substrates for the CSR process (Chaudhuri and Alt, 2004). We used qPCR to determine germline transcript expression directed by the I promoter of each isotype. Total RNA was prepared at day 4 from activated *c-myc*$^{fl/+}$ and *c-myc*$^{fl/fl}$ B cells and was reverse-transcribed and amplified using isotype-specific GLT primers. We found that *c-myc*$^{fl/fl}$ B cells expressed normal Iγ1-Cγ1 germline transcript levels in response to anti-CD40 plus IL-4 compared to *c-myc*$^{fl/+}$ cells (Fig 17A).). We detected low expression levels of other germline transcripts of different isotypes (two-fold reduction in Iε-Cε, five-fold reduction in Iγ3-Cγ3, and two-fold reduction in Iγ2b-Cγ2b

germline transcripts) in *c-myc*$^{fl/fl}$ compared to control B cells (Fig 17A, B). We conclude that c-Myc-deficient B cells express normal Iγ1-Cγ1 germline transcript levels but show impairment in the expression of Iε-Cε Iγ3-Cγ3 and Iγ2b-Cγ2b germline transcripts after *in vitro* stimulation).

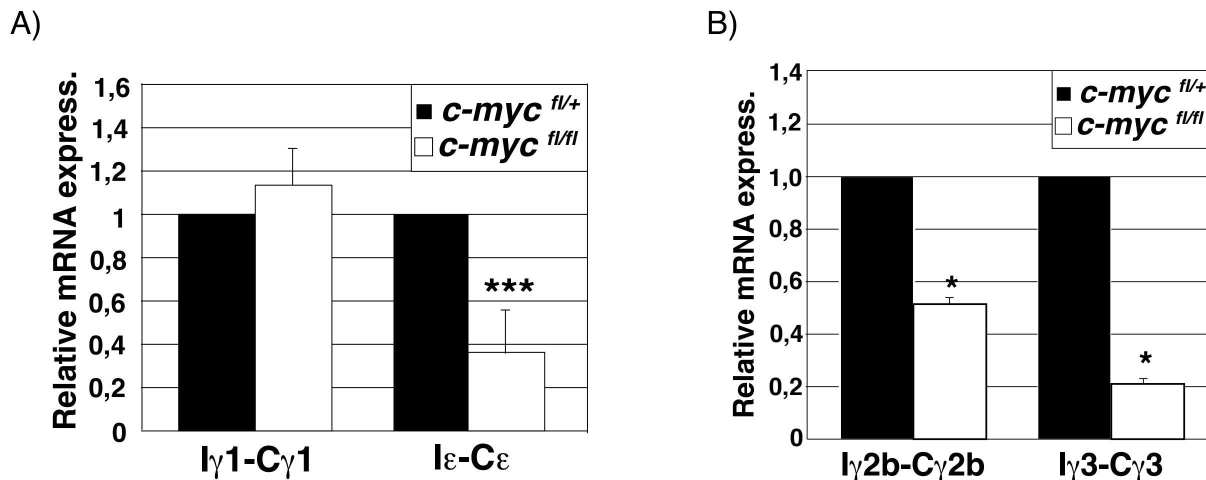


Fig 18. Germline transcript expression. qPCR of germline transcripts from sorted B220^{+}GFP^{+} B cells from *myc* $^{fl/+}$ and *c-myc* $^{fl/fl}$ mice, activated with anti-CD40 plus IL-4 (A) or LPS (B); *n* = 6, **p*>0.05 *and* ****p*>0.001.

5.4. Lack of *aicda* induction in *in vitro*-activated c-Myc-deficient B cells

Activation-induced cytidine deaminase, *aicda* (AID) has a critical role in both somatic hypermutation (SHM) and class switch recombination (CSR) (Muramatsu et al. 2000). AID is expressed by activated B cells, mainly in germinal centers (GC) of peripheral lymphoid organs (Honjo et al., 2002; Honjo et al., 2004) and initiates SHM and CSR by deaminating dC residues to yield dU:dG mispairs in DNA (Neuberger et al., 2003; Peled et al., 2008). These U:G mispairs trigger DNA repair processes that introduce mutations in V(D)J regions or DNA breaks, including double-stranded DNA breaks, and lead to non-classic non-homologous end-joining and CSR.

We measured *aicda* transcription levels in in c-Myc-deficient B cells to test whether the failure in CSR could be due to a problem in *aicda* induction. We sorted B220^{+}GFP^{+} B cells from *c-myc*$^{fl/+}$ and *c-myc*$^{fl/fl}$ mice and activated them *in vitro* with anti-CD40 plus IL-4 for 4 days. cDNA was analysed by qPCR with primers specific for *aicda* and *c-*

myc genes. We found that transcriptional levels of *aicda* were highly downregulated in c-Myc-deficient B cells compared to controls (21-fold compared to control) (Fig 18). We concluded that c-Myc-deficient B cells do not express *aicda*.

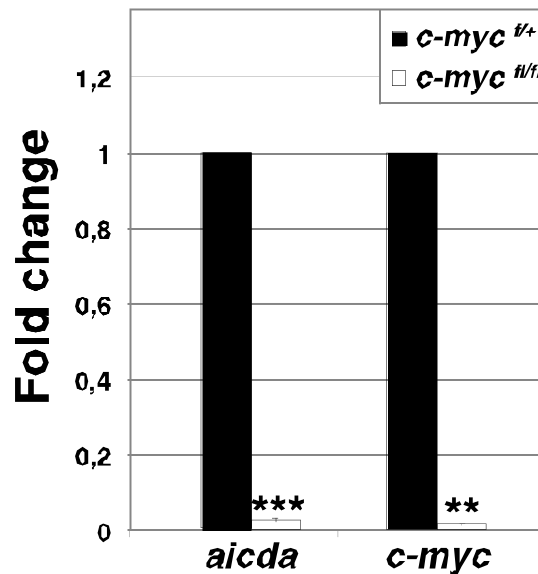


Fig 19. *aicda* downregulation in c-Myc-deficient B cells. Sorted $B220^{+}GFP^{+}$ mature B cells from *c-myc*$^{fl/fl}$ and *c-myc*$^{fl/+}$ mice were activated with anti-CD40 plus IL-4. Control (black) and mutant cells (white). Results were normalized to β-actin, $n = 6$. **p>0.01 *and* ***p>0.001.

5.5. c-Myc activates *aicda* expression through cytokine-responsive region 4

The *aicda* locus has four regions, which are differentially bound by many transcription factors and regulate transcription in a cell-specific manner (Yadav et al., 2006). The first region (R1) is located ~1 kb upstream of exon 1 and contains the minimal promoter. It has sites for Stat6, Sp1, NF-κB, HoxC4 and Pax5 (Dedeoglu et al., 2004; Gonda et al., 2003; Park et al., 2009; Yadav et al., 2006). The second region (R4) is found ~8 kb upstream of exon 1 in the mouse, and contains predicted binding sites for NF-κB, Stat6, C/EBP and Smad3/4 proteins (Tran et al., 2010; Yadav et al., 2006). This region responds to stimulation by anti-CD40 antibody and LPS, and upregulates *aicda* expression. The third region is located in the first intron and has sites for negative and specific B cell regulatory factors (E proteins and Pax5) (Gonda et al.,

2003; Sayegh et al., 2003; Tran et al., 2010; Yadav et al., 2006). The fourth region is located ~6 kb downstream of exon 5 in the mouse (Tran et al., 2010; Yadav et al., 2006) and appears to act as an enhancer (Crouch et al., 2007).

We analysed *aicda* genomic locus and identified a conserved putative c-Myc regulatory binding site (E-box) within cytokine-responsive region 4 (R4). To ensure the minimal activation of the promoter, we cloned the different regions, together with R1, into the pGL3-luc reporter vector (Fig 20A) and cotransfected them into the M12 AID-expressing B cell line, with different amounts of the *pRV-IRES-gfp-c-myc* expression vector. We subsequently measured luciferase activity and found c-Myc dose-dependent activation in the *R1.R4* construct, which contains the E-box within the *R4* cytokine-responsive region; in the *R1.R4.I1* construct, which includes the *I1* inhibitory region, this activation was abolished. There was no response in other constructs (Fig 19B).

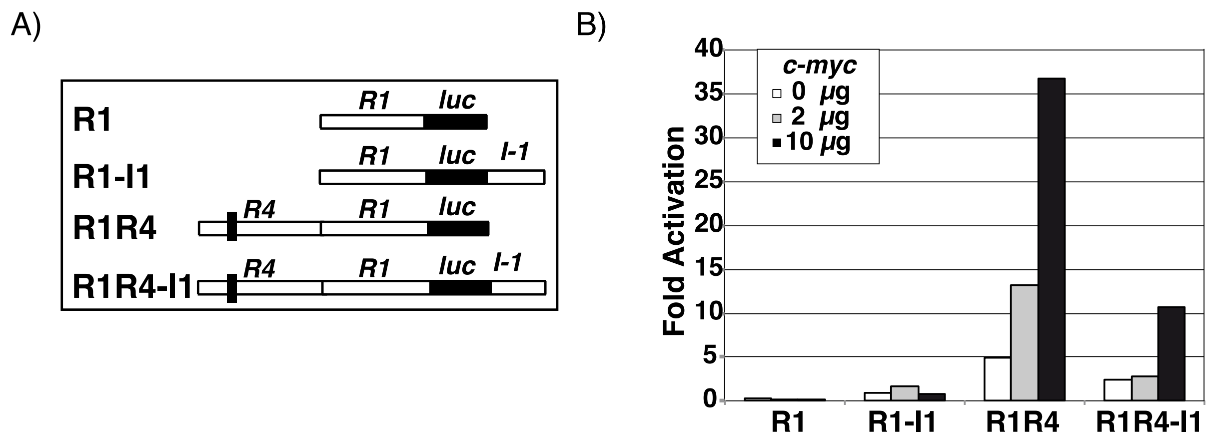


Fig 20. c-Myc activates *aicda* cytokine-responsive region 4 . A) Scheme showing the constructs tested in B). Black boxes in R4 indicate E-box. B) Luciferase reporter assays of constructs in A) in the M12 B cell line. Data are representative of two experiments.

5.6. c-Myc binds to a genomic fragment containing region 4 *in vivo*

To determine whether c-Myc binds to the R4 region, we used a ChIP assay in the M12 AID-expressing B cell line. Immunoprecipitated DNA was amplified using specific primers that flank the E-box within region 4; using PCR, we observed four-fold enrichment of the DNA fragments immunoprecipitated with a c-Myc-specific antibody compared to pre-immune sera (Fig 21). These data indicate that c-Myc activates *aicda* expression by binding to R4 containing the E-box.

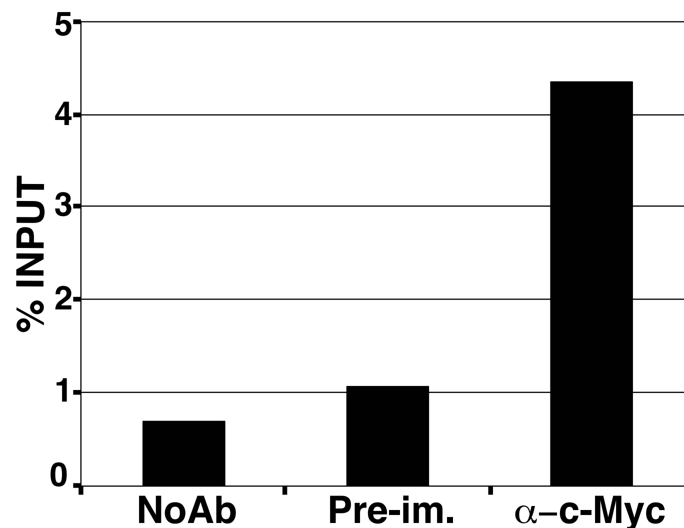


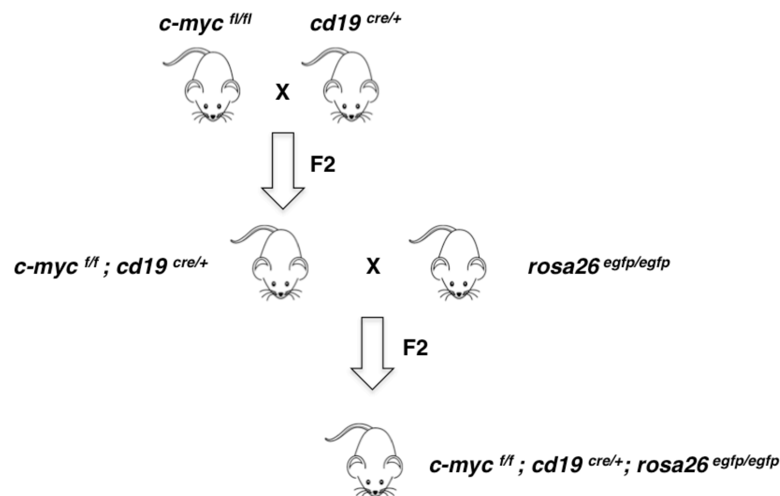
Fig 21. Chromatin immunoprecipitation in M12 cells. Immunoprecipitation was performed with anti-c-Myc antibody or pre-immune serum as negative control (see Methods). Data are representative of three independent experiments.

5.7. *In vivo* rescue of class switch recombination in *IgK-AID* c-Myc-deficient B cells

To determine whether *aicda* expression is sufficient to rescue class switch recombination in c-Myc-deficient B cells, we crossed the IgK-AID mouse (Robbiani et al., 2009) with *cd19$^{cre/+}$c-myc$^{fl/fl}$rosa26$^{egfp/egfp}$* mice (see above). The resulting mice had c-Myc-deficient AID transgenic B cells in which ectopic expression of AID was controlled by immunoglobulin *kappa* chain enhancers (Fig 22A). We isolated B220^{+}GFP^{+} B cells from *cd19$^{cre/+}$c-myc$^{fl/+}$rosa26$^{egfp/egfp}$* (*c-myc$^{fl/+}$*), cd19$^{cre/+}$c-

myc$^{fl/fl}$;*rosa26*$^{egfp/egfp}$ (*c-myc*$^{fl/fl}$) and *cd19*$^{cre/+}$*c-myc*$^{fl/fl}$;*rosa26*$^{egfp/egfp}$*IgK-AID* (*c-myc*$^{fl/fl}$*IgK-AID*) mice, cultured them with anti-CD40 plus IL-4, and analysed the percentage of IgG1-expressing cells by FACS at day 4. *c-myc*$^{fl/fl}$*IgK-AID* B cells were able to switch to the IgG1 isotype (8.34%), at difference from *c-myc*$^{fl/fl}$ IgG1^{+} cells(1.92%) (Fig 22B). Using ELISA, we also tested for IgG1 in culture supernatants from these cells and found a seven-fold increase in *c-myc*$^{fl/fl}$*IgK-AID* (0.7 μg/mL) compared to *c-myc*$^{fl/fl}$ (0.1 μg/mL) (Fig 22C). These data shows that ectopic expression of *aicda* in c-Myc deficient B cells is sufficient to rescue class switch recombination.

A)



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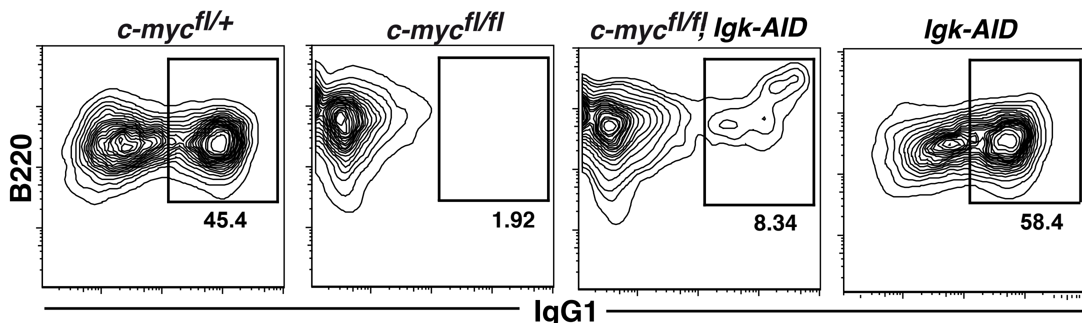

C)

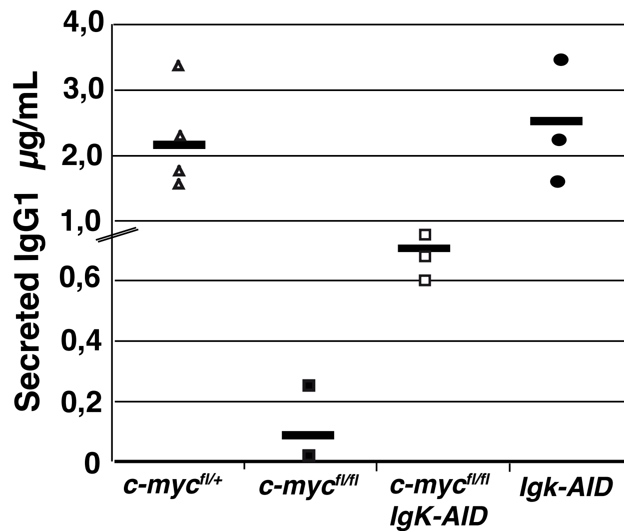

Fig. 22. AID expression restores class switch recombination in c-Myc-deficient B cells. A) Scheme showing the *cd19$^{cre/+}$;c-myc$^{fl/+}$;rosa26$^{egfp/egfp}$* mouse model. B) Flow cytometry analysis of surface IgG1 expression in *cd19$^{cre/+}$;c-myc $^{fl/+}$;rosa26$^{egfp/egfp}$* (*c-myc$^{fl/+}$*), *cd19$^{cre/+}$;c-myc$^{fl/fl}$;rosa26$^{egfp/egfp}$* (*c-myc$^{fl/fl}$*) *and cd19$^{cre/+}$;c-myc$^{fl/fl}$;rosa26$^{egfp/egf}$ IgK-AID* (*c-myc$^{fl/fl}$;IgK-AID*) B cells, activated with anti-CD40 plus IL-4 for 4 days. Data are representative of at least three independent experiments. C) ELISA for IgG1 in supernatants from B cells in B). n = 3, *p>0.05.

5.8. Impairment of proliferation in activated c-Myc-deficient B lymphocytes

The number of cell divisions is correlated with CSR (Hodgkin et al., 1996) and with AID expression (Rush et al., 2005). Cells must carry out at least one replication round to express a switched isotype. This process is associated with accessibility of the immunoglobulin locus. Previous results with c*-myc$^{fl/fl}$cd19$^{cre/+}$* B cells showed that proliferation is impaired in c-Myc-deficient activated B cells (de Alborán et al., 2001). We monitored cell division with the dye CellTrace Violet (similar to CFSE) by flow cytometry analysis of fluorescence decay, and observed that activated *c-myc$^{fl/fl}$ and c-myc$^{fl/fl}$;IgK-AID* B cells did not undergo even a single division (Fig 23A), at difference from *c-myc$^{fl/+}$* control cultures. These results also correlated with cell counts for these cultures after treatment with anti-CD40 plus IL-4 or LPS at day 4 (Fig 23B).

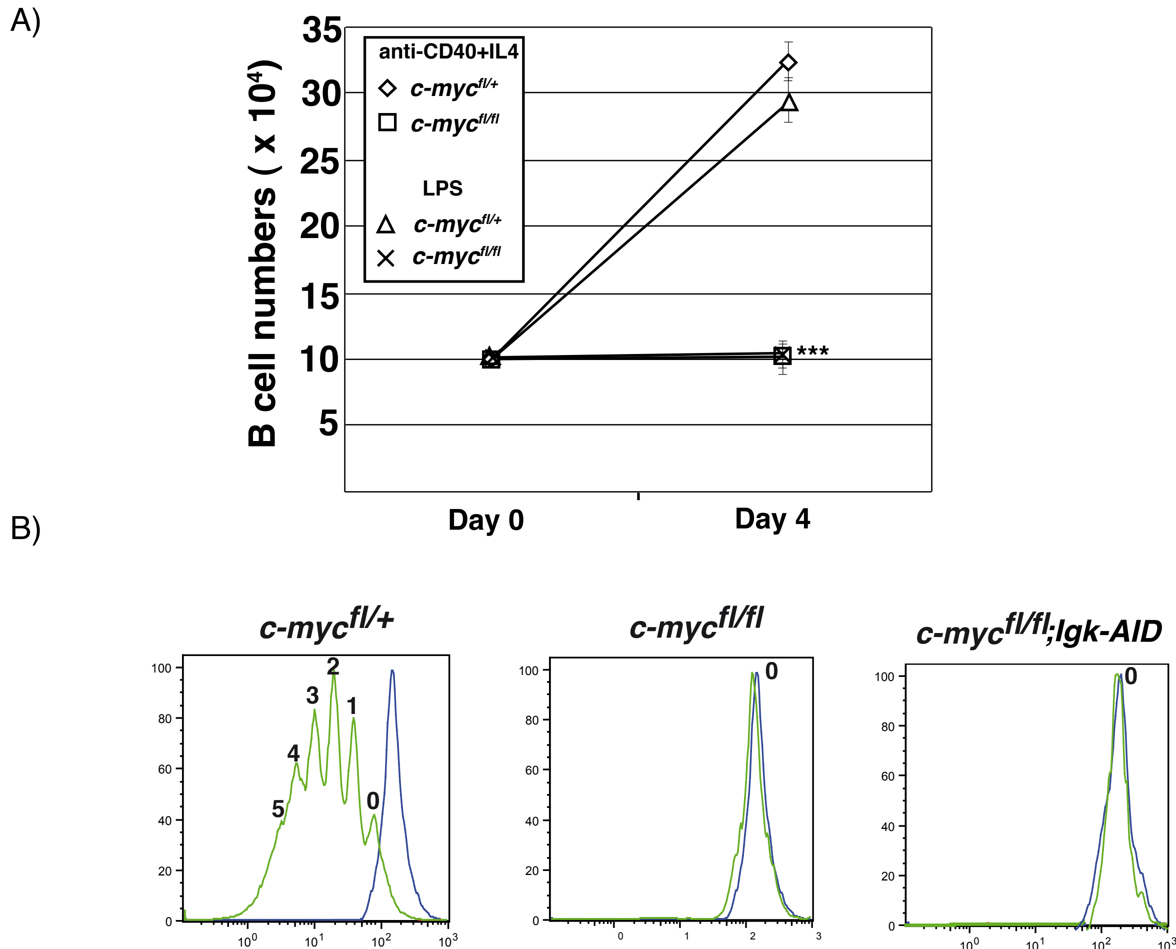

Fig 23. Activated c-Myc-deficient B cells do not proliferate. Sorted B220$^+$GFP$^+$ spleen B cells from *c-myc*$^{fl/+}$, *c-myc*$^{fl/fl}$, and *c-myc*$^{fl/fl}$*;IgK-AID* mice were stained with CellTrace Violet, followed by anti-CD40 plus IL-4 activation. Cells were counted A) and analysed by flow cytometry four days later B). n = 6, ***p>0.001

5.9. Class switch recombination in *c-myc*$^{fl/fl}$*;IgK-AID* B cells correlates with circle transcript expression

Class switch recombination generates switched DNA circles that originate isotype-specific transcripts from I promoters, termed "circle transcripts" (CT). CT are produced in cells that not only express AID but also undergo class switch recombination (Kinoshita et al., 2001). We tested whether the CSR rescue observed in FACS and ELISA correlated with CT expression (Iγ-Cμ) in B cells cultured from *c-myc*$^{fl/fl}$*;Igκ-AID* mice. We sorted B220$^+$GFP$^+$ spleen B cells from *c-myc*$^{fl/+}$*, c-myc*$^{fl/fl}$ and *c-myc*$^{fl/fl}$*;IgK-*

AID, AID-deficient (*AID*$^{-/-}$), and AID-deficient AID-transgenic (AID$^{-/-}$;IgK-AID) mice and cultured them with anti-CD40 plus IL 4 for 48 h. We then re-sorted them by GFP expression to rule out possible contamination with non-c-Myc-deficient B cells (GFP-negative) and analysed Iγ-Cμ CT and *aicda* expression by qPCR. We observed a 10-fold increase in Iγ-Cμ transcripts in *c-myc*$^{fl/fl}$*; Igκ*-AID compared to *c-myc*$^{fl/fl}$ B cells (Fig 24). We also included AID$^{-/-}$ and AID$^{-/-}$; IgK-AID B cells as negative and positive controls of rescue, respectively. These results showed that AID expression in c-Myc-deficient B lymphocytes is sufficient for IgG1 switching after *in vitro* anti-CD40 plus IL-4 activation, in the absence of cell division

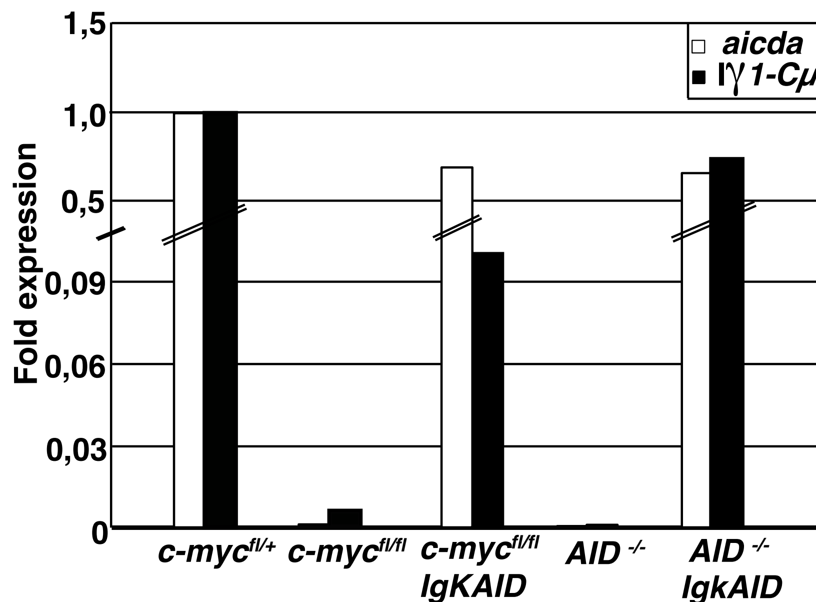

Fig. 24. Circle transcript expression in activated B cells. Spleen B cells from *c-myc*$^{fl/+}$, *c-myc*$^{fl/fl}$, *c-myc*$^{fl/fl}$*;IgK-AID*, *AID*$^{-/-}$ and AID$^{-/-}$;IgK-AID mice were sorted by B220^{+}GFP^{+} expression, activated with anti-CD40 plus IL-4, and re-sorted after 48 h by GFP expression. cDNA was analysed by qPCR with specific primers to amplify Iγ-Cμ and *aicda* transcripts. Data show x-fold change relative to *c-myc*$^{fl/+}$ and are representative of two independent experiments.

6. DISCUSSION

6. **Discussion**

Although the role of the proto-oncogene *c-myc* in the cell cycle and apoptosis has been studied extensively, its function in B cell development remains poorly understood, probably due to the lack of suitable mouse models of *c-myc* inactivation in developing B cells.

In this study, we shed light on the role of the proto-oncogene *c-myc* in early stages of B lymphocyte differentiation, as well as during the critical process of B cell activation and class switch recombination through specific activation of target genes. Using two constitutive conditional mouse models in which *c-myc* is inactivated at different times, we attempted to determine whether c-Myc has a function in B lymphocyte development. In the first model, we bred *c-myc*$^{fl/fl}$ with *mb-1*$^{cre/+}$ mice, in which Cre recombinase expression is controlled by the *Ig*α promoter (*mb-1*), inactivating *c-myc* at early steps of B cell differentiation (Vallespinós et al., 2011, in press). In the second model, we used a *c-myc*$^{fl/f}$*;cd19*$^{cre/+}$ mouse to delete *c-myc* specifically in mature B lymphocytes, and crossed it with a *rosa26*$^{egfp/egfp}$ reporter mouse, to distinguish between deleted and non-deleted B cells (Baena et al., 2005)

To study the role of *c-myc* in early B lymphocyte differentiation and to define the molecular mechanism by which c-Myc controls these process, our starting point was the observation in our laboratory that *c-myc*$^{fl/fl}$*;mb-1*$^{cre/+}$ mice showed a blockade in B cell development at the pre- (B220$^+$CD25$^+$) to immature (B220^{low}IgM$^+$) B cell stages (Fig 9A). Pro-B cells appeared to be less severely affected, probably due to the time at which *c-myc* deletion occurs. These results correlated with the absence of c-Myc-deficient mature B cells in c-*myc*$^{fl/fl}$*;mb-1*$^{cre/+}$ mouse spleens (Fig 9B), since the small percentage of B220$^+$IgM$^+$ B cells in spleen were not deleted (not shown) and could thus be considered wild type cells. A similar blockade was described in *mb-1-cre*-dependent deletion of the *c-myb* gene (Fahl et al., 2009), considered a transcriptional regulator of *c-myc* (Kumar et al., 2003). Our results are thus in agreement, and place c-Myc in the context of c-Myb B cell regulation via Ebf1.

In addition to its role in *ebf1* regulation, c-Myc is implicated in the control of apoptosis and proliferation of pro- and pre-B cell populations (Vallespinós et al., 2011). To determine whether c-Myc acts in B cell development by modulating specific B cell genes, we analysed the expression of transcription factors known to regulate B lineage and commitment (O'Riordan and Grosschedl, 1999). We found a decrease in *e2a, ebf1*, and *pax5* transcripts from *c-myc*$^{fl/fl}$*;mb-1*$^{cre/+}$ pro-B cells compared to controls; these decreases were more notable in the pre-B cell compartment, due to the time of deletion. These transcription factors are important regulators of B lymphocyte differentiation (Bartholdy and Matthias, 2004; Medina et al., 2004) and the knock-out mouse models also show a blockade at similar stages of B cell differentiation compared to c*-myc*$^{fl/fl}$*;mb-1*$^{cre/+}$ mice (Bain et al. 1994; Lin and Grosschedl 1995). Our results therefore indicated a specific role for c-Myc in the regulation of the early B cell differentiation pathway.

We observed no c-Myc transcriptional regulation of *e2a* or *pax5* constructs in luciferase reporter assays (Fig 12); it is nonetheless possible that there is an indirect effect of c-Myc on their regulation. Downregulation of *ebf1* is described to negatively affect *e2a* expression levels, since *ebf1*$^{-/-}$ showed two-fold downregulated *e2a* transcripts, indicating a positive feedback loop (Zhuang et al., 2004). *pax5* downregulation in c*-myc*$^{fl/fl}$*;mb-1*$^{cre/+}$ pre-B cells can be explained by the downstream position of *pax5* relative to *ebf1* in the activation pathway (O'Riordan et al., 2004; Medina et al., 2004; Roessler et al., 2007).

In the case of the *ebf1*α construct, we found transcriptional activation in a c-Myc dose-dependent manner when we assayed it in a human fibroblast cell line (HEK 293T) and an L1-2 murine pre-B cell line (Fig 13). Moreover, activation was completely abolished when we specifically mutated the E-box 5 (E5). Using ChIP and EMSA assays, we demonstrated that this E-box was directly implicated in the *ebf1*transactivation process, through c-Myc binding (Fig 14 and 15, respectively).

The *ebf1* gene has two promoters, *ebf1*α and *ebf1*β, which are differentially regulated in a hierarchical network in which E2a activates *ebf1* expression by binding to the

ebf1α promoter; Ebf1 activates *pax5* expression and Pax5 maintains *ebf1* levels through activation of the *ebf1β* promoter (Roessler et al., 2007). The contribution of the *ebf1α* promoter to total *ebf1* transcript levels is small compared to that of the β promoter; however, we consider that this contribution is essential at early stages of B cell differentiation, due to the complex regulation of *ebf-1* expression (Roessler et al,. 2007). A small amount of *ebf1* (transcribed from the *ebf1α* promoter) triggers *pax5* expression, which in turn induces expression of large amounts of Ebf1 by activating the *ebf1β* promoter. In addition, Ebf1 has a role in *e2a* activation, as *ebf1*$^{-/-}$ B cells show downregulated *e2a* expression (Zhuang et al., 2004). We therefore propose that c-Myc, together with E2a, activates *ebf1* (through the *ebf1α* promoter), which in turn activates *pax5*, thus triggering and maintaining the B cell differentiation program.

These results identified *ebf1* as a c-Myc target gene and illustrate a novel c-Myc function in the complex regulation of B lymphocyte development.

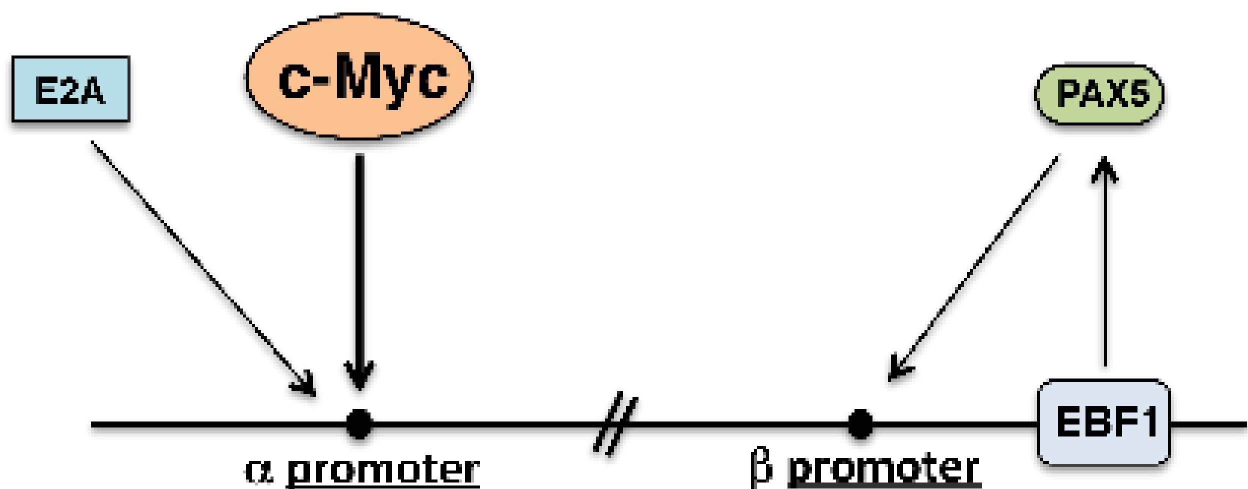

Fig 25. Model of *ebf1* regulation through c-Myc.

In many cell types, including B lymphocytes, c-myc expression must be downregulated to achieve terminal differentiation (Lachman and Skoultchi, 1984; Lin et al., 2000). In B cells, however, this event is preceded by an increase in its expression. To study the role of c-Myc in CSR, we used a c-mycfl/fl;cd19cre/+;rosa26egfp/egfp mouse model and analysed the behaviour of c-Myc-deficient B cells in culture after activation with anti-CD40 plus interleukin-4 or LPS.

We observed severe CSR impairment in c-Myc-deficient cells compared to controls (Fig 16A, B). We tested the activation status of these cells and confirmed expression of activation markers such as CD69 (de Alborán et al., 2001). c-Myc-deficient B cells were therefore able to trigger the pathway that gives rise to expression of these markers at the membrane, and impairment was thus due to the implication of c-Myc in some step of the class switch recombination pathway.

The class switch recombination process is triggered by specific cytokine stimulation, which promotes transcription through an intronic promoter (I) and gives rise to a so-called sterile germline transcript (GLT) (Berton et al., 1989). Synthesis of this GLT is absolutely necessary for correct development of the CSR process (Islam et al., 1994), and has been correlated with accessibility of the C_H locus to the switch machinery (Stavnezer et al., 2000). Abolishment of this transcript severely affects CSR (Jung et al., 1993; Zhang et al., 1993).

We analysed GLT expression in the c-Myc-deficient B cells to determine whether the CSR impairment was due to a failure in GLT expression. As shown in Fig 18, c-Myc-deficient B cells expressed normal levels of Iγ-Cγ GLT, but the other GLT (such as Iγ2b-Cγ2b, Iγ3-Cγ3 and Iε-Cε appeared to be downregulated compared to controls. Since GLT expression levels correlated with CSR efficiency (Lee et al., 2001), we further analysed the IgG1 impairment in activated c-Myc-deficient B cells expressing Iγ-Cγ GLT, which were unable to carry out the CSR process.

CSR is a cell proliferation-coupled mechanism. Activated B cells must undergo a certain number cell divisions to switch from IgM to another isotype (Hodgkin et al., 1996; Hasbold et al., 1998; Hasbold et al., 1999). We tested the ability of c-Myc-deficient B cells to proliferate in the presence of anti-CD40 plus IL-4 by CFSE-like Violet CellTrace staining, and found that *c-myc*$^{fl/fl}$ mouse B cells did not proliferate for even a single cell division (Fig 23). The proliferation requirement correlates with expression of an enzyme critical for CSR, activation-induced cytidine deaminase (*aicda* and the protein AID) (Muramatsu et al., 1999). AID acts as a DNA deaminase, deaminating dC residues to dU in single-stranded DNA in the non-template DNA strand within the R-loop structure inside transcribed S region repeats (Xu et al., 2005). AID expression is absolutely necessary for CSR (Muramatsu et al., 2000) and its expression levels increase with the number of cell divisions (Rush et al., 2005). To test whether AID levels were diminished, affecting CSR in c-Myc-deficient B cells, we analysed *aicda* expression in activated *c-myc*$^{fl/fl}$ B cells and observed a 10-fold decrease compared to control cells (Fig 19). This downregulation in *aicda* levels might thus reflect direct c-Myc regulation.

The mechanisms that control AID expression are highly regulated, and AID deregulation results in translocations and malignant transformation of B cells (Ramiro et al., 2006; Kuppers et al., 2001) and T cells (Okazaki et al., 2003). Nonetheless, the molecular pathways that govern the transcriptional regulation of *aicda* have not been completely elucidated. Following B cell activation *in vivo* or *in vitro* through CD40 and the IL-4 receptor, a potent cascade of intracellular signal transduction pathways triggers *aicda* induction (Muramatsu et al., 1999; Jabara et al., 2002; Schrader et al. 2005) through various transcription factors such as Pax5 (Gonda et al., 2003), E2a (Sayegh et al., 2003), Stat6, NF-κB (Dedeoglu et al., 2004) and HoxC4 (Seok-Rae et al., 2009). Other negative modulatory factors such as Blimp1 and Id2 downregulate *aicda* and restrict its expression primarily to germinal centre B cells. Our results identified *aicda* as a previously unreported c-Myc target gene and correlated the need for cell proliferation with CSR.

To determine whether AID restoration was sufficient to restore CSR in c-Myc-deficient

B cells, we bred *c-myc*$^{fl/fl}$*;cd19*$^{cre/+}$*;rosa26*$^{egfp/egfp}$ with *IgK-AID* transgenic mice and used FACS to analyse IgG1 expression. We also tested expression of circle transcripts as a hallmark of CSR (Kinoshita et al., 2001) and found 10-fold increased levels of Iγ1-Cμ expression transcripts in *c-myc*$^{fl/fl}$;*IgK-AID* B cells compared to controls.

Our findings showed partial rescue of IgG1 expression in AID-expressing c-Myc-deficient B cells (Fig 22) in the absence of proliferation, and suggest a prominent role for c-Myc in the generation of a normal, potent immune response. c-Myc is required not only for cell proliferation, but also for differentiation and CSR. Our data show that cell proliferation and CSR are linked physiologically by c-Myc, although these functions can be uncoupled mechanistically. The results highlight the fact that CSR can occur in the absence of proliferation, and that the mechanisms by which both processes are linked is controlled by c-Myc. We thus postulate that c-Myc controls *aicda* expression while promoting cell proliferation, providing a fine-tuning mechanism to balance specific cell expansion and enhancement of a secondary immune response.

7. **CONCLUSIONS**

7. Conclusions

1) Conditional inactivation of *c-myc* in developing B lymphocytes causes a blockade at the pro- to pre-B cell transition in *c-myc*$^{fl/fl}$;*mb-1*$^{cre/+}$ mice.

2) c-Myc-deficient pro- and pre-B cells show downregulated expression of specific B cell transcription factors genes such as *e2a, ebf1* and *pax5.*

3) c-Myc controls early stages of B lymphocyte differentiation through binding to the *ebf1*α promoter, and activating transcription of *ebf-1.*

4) c-Myc-deficient B lymphocytes do not undergo CSR after in vitro activation.

5) Activated c-Myc-deficient B lymphocytes express the CD69 activation marker, normal levels of Iγ1-Cγ1 germline transcripts and do not express *aicda.*

6) c-Myc activates *aicda* transcription through binding to the R4 regulatory region.

7) Ectopic expression of aicda in c-Myc-deficient B lymphocytes is sufficient to rescue CSR.

8) CSR can occur in the absence of cell division.

8. **RESUMEN (ESPAÑOL)**

8. **RESUMEN**

8.1. Introducción

El proto-oncogen c-myc fue descubierto como el homologo celular del oncogén v-myc, perteneciente al retrovirus MC29.

La proteina c-Myc forma parte de la familia de factores de transcripción bHLHZ implicados en la regulación de la proliferación, apoptosis y diferenciación celular (Blackwell et al., 1990; Landshulz et al., 1988; Murre et al., 1989).

La expresion de c-myc está altamente regulada tanto a nivel transcricional como post-transcripcional (Jones and Cole, 1987; Brewer and Ross, 1988). Su expresión es casi indetectable en células quiescentes sin embargo, tras la exposición a diferentes mitógenos, se produce un aumento significativo y con ello, la célula comienza la transición G0/G1 hacía fase S (Kelly et al., 1983; Campisi et al., 1984). Una caracteristica típica de c-myc es que una desregulación de su expresión favorece la formación de tumores *in vivo* (Adams and Cory, 1985; Morgenbesser and DePinho, 1994). Sólo en EEUU se estima que 1/7 de los canceres humanos que causan muerte al año, presentan alteraciones en la regulación de c-myc.

c-Myc dimeriza con la proteina Max y forma un complejo protéico que se une al ADN a traves de la secuencia CACGTG denominada caja E y activa la transcripción de sus genes diana (Blackwoodand and Eisenman, 1991; Ayer and Eisenman, 1993; Amati and Land, 1994).

Tambien se ha descrito la capacidad de c-Myc para inhibir la transcripción de numerosos genes, muchos de ellos, implicados en la salida de ciclo celular (Gartel and Shchors, 2003).

Además del importante papel desempeñado por c-Myc en proliferación y apoptosis, no menos importante, es su función en diferenciación celular.

c-Myc se expresa normalmente en células inmaduras en proliferación y una disminución en su expresión está vinculada con el desencadenamiento de la diferenciación celular (Chang et al., 2000; Henriksson and Luscher, 1996). Sin embargo, la expresion ectópica de *c-myc* bloquea la diferenciación terminal ne muchos tipos celulares, tanto *in vivo* como *in vitro* (Facchin and Facchin, 1998; Iritani and Eisenman, 1999). Un ejemplo bien caracterizado de ello es el proceso de diferenciación terminal de las células B, en el cual la activación de *blimp-1*, cuya función en diferenciación B esta bien descrito, provoca una disminición de los niveles de expresión de *c-myc* (Lin et al., 1997).

Durante el proceso de diferenciación de las células B, tienen lugar en una serie de eventos característicos, tales como, la expansión de unos pocos precursores en un gran número de células B inmaduras, que emigran a la periferia para formar parte del repertorio de células B maduras (Hardy and Hayakawa, 2001) ; el reordenamiento de los genes que codifican para el receptor de células B (Davis and Bjorkman, 1988) y el silenciamiento de aquellas células que expresan receptores afines a antígenos propios. El desarrollo de células B maduras desde los precursores hematopoyéticos necesita de la acción coordinada de una serie de factores de transcripción, tales como Pu.1, E2a, Ebf1 y Pax5. De los cuales los tres últimos son clave en el proceso de diferenciación hacia célula B y del mantenimiento de la identidad B (Zhuang et al., 1994; Lin et al., 1995; Urbanek et al., 1994). Varios trabajos, han mostrado como la expresión de c-myc a lo largo del proceso de diferenciación de linfocitos B, está altamente regulada y ya que su expresión se requiere en estadios concretos (de Alboran et al., 2001). Por tanto, hemos intentado dilucidar los mecanismos moleculares que subyacen a dicha necesidad de c-Myc, mediante el empleo de modelos de inactivación *in vivo* que permiten la inactivación de c-myc en un tipo celular específico mediante el empleo del sistema Cre-Lox.

El principal papel de las células B es la produccextrañas al propio organismo. Dicho pro. Esta productionas co la expansioniento de los genes que codifican para el receptorión de anticuerpos frente a diferentes moléculas extrañas al propio organismo, la cual tiene lugar mediante tres procesos bien diferenciados: La recombinación VDJ de los genes de las inmunoglobulinas, la hipermutación somática de las regiones variables (SHM) y el cambio de clase de isotipo (CSR). Tanto la SHM como el CSR requieren de la expresión de la proteína AID (cuyo gen se denomina *aicda*), la cual está implicada directamente en el proceso de hipermutación y de recombinación, ya que provoca mutaciones y roturas del ADN que conllevan a la alteración de la secuencia en las regiones variables de las inmunoglobulinas y al reordenamiento de las regiones constantes de las mismas, respectivamente. AID desamina las citosinas de las regiones variables y de las secuencias S, convirtiéndolas en uracilos y provocando un desemparejamiento G:U, que son reconocidos por la maquinaria de reparación del ADN y conllevan en ultimo lugar a la introducción de mutaciones (SHM) o a la rotura del ADN y posterior recombinación (CSR).

El proceso de cambio de clase de isotipo (CSR) diversifica la función efectora del anticuerpo sin influir en la afinidad especifica por el antígeno y tiene lugar entre las secuencias repetitivas (S) de los genes que codifican para las regiones constantes (Stavnezer et al., 2000).

Para que se lleve a cabo el cambio de clase, es necesario que exista transcripción a través de la región constante a la cual vaya a darse el proceso de recombinación. Con ello se produce un transcrito (GLT) que no codifica proteínas pero que es fundamental para que el cambio de clase se produzca con normalidad (Shinkura et al., 2003; Yu et al., 2003). Cada no de los promotores de las secuencias S se transcribe de forma diferencial en respuesta a una amplia variedad de mitógenos y citoquinas que dirigen de este modo el cambio de clase hacia uno de los isotipos en particular (Stavnezer, 2000). Uno de los mecanismos que controlan el cambio de clase (CSR), consiste en la regulación específica de la expresión de AID.

Además existe una relación bien documentada entre la proliferación celular y el proceso de CSR, ya que se ha observado un incremento en el proceso de cambio de clase en aquellas células B que han llevado a cabo un mayor numero de divisiones. que la expresión de AID está vinculada al número de divisiones celulares (Tangye and Hodgkin PD., 2004). Esto se explica mediante la unión existente entre los niveles de expresión de AID y el número de divisiones que tiene lugar en una misma célula (Rush et al., 2005). Ya que c-Myc juega un papel crucial en proliferación y que ésta es necesaria para el proceso de CSR, hemos querido profundizar en los mecanismos moleculares que gobiernan dicho proceso, mediante el empleo de modelos de inactivación de c-myc *in vivo.*

8.2. Resultados

Modelos constitutivos de inactivación de *c-myc*

Debido a la letalidad embrionaria del knock-out de *c-myc* en línea germinal de ratón (Davis et al., 1993), hemos utilizado un modelo de inactivación condicional (de Alborán et al., 2001) que nos permite estudiar la función de c-Myc en cada uno de los tipos celulares de interés. En el caso de células B inmaduras, hemos utilizado el modelo *c-myc* $^{fl/fl}$ *mb1* $^{cre/+}$ en el cual inactivamos c-myc específicamente en los estadios de pro y pre-B en médula ósea.

Para llevar a cabo el estudio de la función de c-Myc en el proceso de cambio de clase (CSR), hemos utilizado el modelo *c-myc* $^{fl/fl}$; *cd19* $^{cre/+}$; *rosa26* $^{egfp/egfp}$ en el que la eliminación de *c-myc* se produce en el estadio de célula B madura y conlleva la expresión de GFP como marcador reportero.

c-Myc es necesario para la diferenciación B

Análisis previos en nuestro laboratorio, mostraron que la eliminación condicional de *c-myc* en linfocitos de médula ósea, provoca un bloqueo en la diferenciación desde los estadios de pro-B y pre-B hasta células B inmaduras (Vallespinós et al., 2011), indicando por ello que c-Myc es necesario para la generación de pro-B y pre-B.

Disminución de la expresión de los factores de transcripción *tcfe2a, e2a, ebf1 y pax5* en células B deficientes en c-Myc

A través del análisis de expresión génica de células pro-B y pre-B deficientes en c-Myc, encontramos una disminución en la expresión de los factores de transcripción *e2a, ebf1 y pax5,* los cuales son fundamentales en el proceso de diferenciación y mantenimiento de identidad de células B.

Estudio de los promotores y regiones reguladoras de los factores de transcripción *tcfe2a, e2a, ebf1 y pax5 y ensayos de reportero de luciferasa*

Mediante el empleo de programas informáticos de alineamiento y estudio de secuencias de ADN, encontramos sitios de unión de c-Myc (cajas E), en cada uno de los promotores de los genes *tcfe2a, ebf1 y pax5*.

Para comprobar si c-Myc estaba activando la transcripción de alguno de estos genes mediante la unió a sus promotores, realizamos ensayos de reportero de luciferasa y comprobamos que ni el promotor de *e2a ni el de pax5* eran activados por c-Myc. Sin embargo, el promotor proximal de *ebf1, ebf1*α mostraba una activación del gene de luciferasa dependiente de c-Myc.Con ello podíamos concluir que c-Myc activa la expresión de *ebf1* a través del promotor *ebf1*α.

c-Myc se une a la caja E5 del promotor α del gen *ebf1*

Mediante el empleo de la técnica de co-inmunoprecipitación de cromatina (ChIP) en la línea celular pre-B L1-2, confirmamos que c-Myc se une al promotor *ebf1*α *in vivo*. Mediante el uso de la técnica de EMSA comprobamos como c-Myc se une específicamente a la caja E 5 contenida en el promotor distal del gen *ebf1*.

Así pues, podemos afirmar que c-Myc regula directamente la expresión de *ebf1* mediante la unión a una caja E situada en su promotor distal (*ebf1*α).

Linfocitos B deficientes en c-Myc no llevan a cabo el proceso de cambio de clase (CSR) *in vitro*

Para estudiar el papel que desempeña c-Myc en la diferenciación terminal de los linfocitos B, aislamos células B maduras GFP^+ $B220^+$ de bazos de ratones *c-myc* $^{fl/+}$ y *c-myc* $^{fl/fl}$ y las activamos en presencia de anti-CD40 más interleuquina 4 o de LPS. Tras 4 días en cultivo, analizamos la expresión de IgG1 y de IgG2b en membrana y comprobamos que las células deficientes en c-Myc no expresaban ninguno de los dos isotipos a los cuales dan lugar ambas activaciones.

También comprobamos, la expresión de marcadores de activación en estas células y observamos que las células *c-myc* $^{fl/fl}$ expresan niveles normales de CD69 comparado con las células control.

Los linfocitos B activados deficientes en c-Myc expresan niveles normales de "germline transcripts" de IgG1

El cambio de clase están regulado por diferentes citoquinas que determinan la transcripción de un determinado promotor de una de las regiones S, dando lugar al denominado "germline transcript" (GLT) (Stavnezer et al., 1986; Lee et al., 2001; Chaudhuri y Alt, 2004).

Analizamos mediante qPCR la expresión de GLT en las células deficientes en c-Myc y comprobamos que tenían niveles similares de IgG1 comparado con las células control. Sin embargo, presentaban niveles disminuidos de otros GLT.

Ausencia de la expresión de *aicda* en linfocitos B deficientes en c-Myc y activados *in vitro*

Aicda desempeña un papel fundamental en los procesos de hipermutación somática (SHM) y de cambio de clase (CSR) (Muramatsu, M. et al. 2000).

Se expresa en células B activadas, principalmente en los centros germinales de los órganos linfoides periféricos (Honjo et al., 2002; Honjo et al., 2004).

Para comprobar si el fallo en CSR en las células deficientes en c-Myc era debido un problema en la expresión de *aicda,* aislamos células B de ratones *c-myc* $^{fl/+}$ *y c-myc* $^{fl/fl}$ y las activamos in vitro durante 4 días. Posteriormente analizamos por qPCR la expresión de aicda y observamos que los linfocitos deficientes en c-Myc no expresaban aicda.

c-Myc activa la expresión de *aicda* a través de la unión a una región reguladora (R4) implicada en la respuesta a citoquinas

Aicda contiene 4 regiones implicadas en su regulación transcripcional. (Yadav et al., 2006). De todas ellas, las regiones 4, que responde a estímulos de citoquinas y 2,implicada en la especificidad de célula B y en la unión de factores represivos, fueron las que presentaron sitios de unión de c-Myc. Mediante ensayos de reportero de luciferasa, comprobamos que la región 4 era capaz de activar la transcripción en repuesta a c-Myc. Para asegurarnos que c-Myc estaba uniéndose a la región reguladora de *aicda*, realizamos ensayos de ChIP y observamos que c-Myc se une específicamente a una fragmento de ADN que contiene una caja E en la región 4.

Rescate in vivo del CSR en células B de ratones IgK-AID deficientes en c-Myc

Para determinar si la mera expresión de aicda era suficiente para llevar a cabo el proceso de cambio de clase en células deficientes en c-Myc, cruzamos los ratones *cd19* $^{cre/+}$ *c-myc* $^{fl/fl}$*; rosa26* $^{egfp/egfp}$ con ratones transgénicos que expresaban la proteína AID bajo el control reguladores positivos de la cadena *IgK*.

Aislamos células B de estos ratones y las activamos *in vitro* con anti-CD40 más interleuquina 4. Observamos un incremento de 4 veces en la cantidad IgG1 en la células deficientes en c-Myc, que expresaban AID de manera transgénica, con respecto a las células deficientes en c-Myc.

Además, corroboramos por ELISA que estas diferencias en la producción de IgG1 eran reales.

Las células B deficientes en c-Myc activadas *in vitro*, no son capaces de proliferar

El numero de divisiones celulares se ha correlacionado con el proceso de CSR (Hodgkin et al., 1996) y con la expresión de AID (Rush et al., 2005). Además se ha comprobado que las células necesitan llevar a cabo al menos una ronda de replicación para expresar niveles normales de GLT y para expresar en membrana una inmunoglobulina determinada.

Ya que estudio previos mostraban que las células B deficientes en c-Myc y activadas in vitro, presentaban un fallo en el proceso de proliferación, quisimos comprobar hasta que grado las células eran incapaces de dividirse. Para ello, las marcamos con un reactivo análogo al CFSE (Violet Cell trace) que nos indica el numero de divisiones que lleva a cabo cada célula en cultivo tras ser activadas con anti CD40 más interleuquina 4 y observamos que las células deficientes en c-Myc no realizaban ni tan siquiera una división celular durante los 4 días de cultivo.

Pudimos concluir que las células deficientes en c-Myc, a pesar de no proliferar, eran capaces de llevar a cabo el cambio de clase en presencia de anti-CD40 mas interleuquina 4.

Expresión de transcritos circulares (CT) en células B deficientes en c-Myc que expresan AID de manera transgénica activadas *in vitro*

El proceso de cambio de clase (CSR) conlleva la producción de ADN circulares tras la recombinación somática, los cuales expresan transcritos específicos a partir del promotor del isotipo al que la célula haya cambiado de clase. La expresión de estos transcritos es específica y demuestra que el proceso de cambio de clase se ha llevado a cabo (Kinoshita et al., 2001).

Para comprobar que el rescate en la producción de IgG1 era real, analizamos la expresión de CT (Iγ1-C*μ*) en las células *c-myc* $^{fl/fl}$*; IgK IAD* mediante qPCR y observamos un aumento de 10 veces con respecto a las células *c-myc* $^{fl/fl}$.

Todos estos resultados mostraron que la expresión en AID en células deficientes en c-Myc era suficiente para llevar a cabo el cambio de clase hacia IgG1 en ausencia de división celular.

8.3. Conclusiones

1-La inactivación condicional de *c-myc* in linfocitos B en desarrollo, provoca un bloqueo en la transición pro-B a pre-B en ratones in *c-myc* $^{fl/fl}$; *mb-1* $^{cre/+}$

2- Los linfocitos pro-B y pre-B deficientes en c-Myc presentan una disminución en la expresión de los factores de transcripción *e2a, ebf1* and *pax5.*

3- c-Myc controla la diferenciación temprana de linfocitos B mediante la unión al promotor *ebf1a,* que promueve la activación del gene *ebf1.*

4- Los linfocitos B deficientes en c-Myc no llevan a cabo el proceso de cambio de clase de isotipo tras la activación *in vitro*

5- Los linfocitos B deficientes en c-Myc, expresan niveles normales del marcador de activación CD69 y del GLT *Iγ1-Cγ1*, pero no expresan *aicda*

6- c-Myc activa la expresión de *aicda* a través de la unión a su región reguladora 4

7- La expresión ectópica de *aicda* en células deficientes en c-Myc es suficiente para rescatar el cambio de clase.

8- El proceso de cambio de clase puede tener lugar en ausencia de división celular

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10. **APPENDIX**

10. APPENDIX

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ABSTRACT

c-Myc, a member of the Myc family of transcription factors, is involved in numerous biological functions including the regulation of cell proliferation, differentiation and apoptosis in various cell types. Of all its functions, the role of c-Myc in cell differentiation is one of the least understood. We addressed the role of c-Myc in B lymphocyte differentiation. We found that c-Myc is essential from early stages of B lymphocyte differentiation *in vivo*, and regulates this process by providing B cell identity via direct transcriptional regulation of the *ebf-1* gene. Our data show that c-Myc influences early B lymphocyte differentiation by promoting activation of B cell identity genes, thus linking this transcription factor to the EBF-1-Pax-5 pathway.

INTRODUCTION

The generation of mature B lymphocytes from early lymphoid progenitors in the bone marrow (BM) is a well-defined process characterized by several cell stages in which a number of transcription factors play a prominent role (1). In BM, pro-B cells ($B220^+c$-Kit^+) begin sequential rearrangement of the immunoglobulin heavy chain locus gene segments (V, D and J) and differentiate into pre-B lymphocytes ($B220^+CD25^+$). Productive rearrangement of the heavy chain locus triggers light chain rearrangement and cell surface expression of both heavy and light chains (IgM). This process gives rise to immature B cells ($B220^+IgM^+$) that migrate from the BM to secondary lymphoid organs to generate mature B lymphocytes (2).

The transcription factors E2A, EBF-1, and Pax-5 have a critical function in early B cell commitment and differentiation (3). Mouse models of gene inactivation have shown the central role of these transcription factors in these processes. Gene inactivation of *tcfe2a* (4, 5) or *ebf-1* (6) in mice leads to an early block in B cell differentiation, before the onset of immunoglobulin heavy chain rearrangement. Both factors appear to work in synergy to activate B cell-specific B lymphocyte genes, conferring B cell identity on early lymphoid precursors (7). E2A deficient pro-B cells are rescued by ectopic expression of EBF-1 *in vitro* but not by Pax-5 (8). Inactivation of *pax-5* in mice causes a block in early B cell differentiation (9) and impaired V_{distal}-to-DhJh rearrangement (10). Pax-5 regulates the expression of B cell-specific genes *cd19* (11)*, blnk* (12) *cd79a* (13) and represses the expression of genes incompatible with B lymphocyte differentiation (14). Ectopic Pax-5 expression is not capable of promoting B cell differentiation in *ebf-1*$^{-/-}$ progenitors. EBF-1 induces *pax-5* gene expression and activates the B cell transcriptional program (15). Taken together, a model has been proposed in which E2A, EBF-1, and Pax-5 act sequentially to promote commitment to B cell fate (7, 15).

The Myc proteins (N-, L- and c-Myc) are members of a basic region/helix-loop-helix/leucine zipper (bHLHZip) transcription factor family and are involved in many biological functions. All Myc proteins heterodimerize with Max and bind to specific sites on the DNA (E-boxes) to regulate their target genes (16); of all its members, c-

Myc is probably the best studied. In humans and mice, c-Myc deregulation is well established as a primary cause of some cancers. It is estimated that the *c-myc* proto-oncogene is activated in 20% of all human cancers (17). It is expressed in many cell types, as well as in early BM progenitors and during B lymphocyte differentiation (18).

Accumulated *in vivo* and *in vitro* evidence shows that c-Myc participates in regulating cell proliferation, differentiation and apoptosis in many cell settings, including B lymphocytes (16). During cell cycling, c-Myc promotes G0/G1-S transition by activating genes that encode proteins of the cyclin/cdk complexes and by repressing cell cycle inhibitors such as *p21* or *p27* in numerous cell types (19). In murine B cell lymphoma lines, apoptosis induced through the B cell receptor correlates with inhibition of *c-myc* expression (20). In mice, mature B lymphocytes lacking c-Myc show impaired proliferation and elevated levels of the cell cycle inhibitor p27, as well as greater resistance to apoptosis (21, 22). c-Myc overexpression in transgenic mouse B cells leads to rapid lymphoma development and mouse death (23).

Despite numerous studies of c-Myc, little is known about its function in B cell differentiation (24). c-Myc downregulation is associated with cell cycle arrest and terminal differentiation in B lymphocytes and myeloid cells (25-27). Here we address the role of c-Myc in early B lymphocyte differentiation, using several conditional mouse models. Our data provide evidence that c-Myc influences B lymphocyte differentiation through the EBF-1/Pax-5 pathway, thus activating B cell identity genes. Finally, our results place c-Myc in the context of transcription factors required for B lymphocyte differentiation.

MATERIALS AND METHODS

Mice and genotyping. Generation of *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice was described (28). To generate *c-myc*$^{fl/fl}$;*mb1*$^{cre/+}$ mice, *c-myc*$^{fl/fl}$ were bred with *mb1*$^{cre/+}$mice (29) and progeny crossbred to yield homozygous (*c-myc*$^{fl/fl}$;*mb1*$^{cre/+}$) and control mice (*c-myc*$^{fl/+}$;*mb1*$^{cre/+}$ or *c-myc*$^{fl/fl}$;*mb1*$^{+/+}$). c-*myc*$^{fl/fl}$;*mx-cre*$^{+}$ or *c-myc*$^{fl/fl}$;*mb1*$^{cre/+}$ mice were bred with *ik*$^{neo/+}$ mice (30) to generate *ik*$^{neo/+}$;*c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ or *ik*$^{neo/+}$;*c-myc*$^{fl/fl}$;*mb1*$^{cre/+}$ mice, respectively. Progeny were crossed to generate homozygous (*ik*$^{neo/+}$;*c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ *or ik*$^{neo/+}$;*c-myc*$^{fl/fl}$;*mb1*$^{cre/+}$) and control mice (*ik*$^{neo/+}$;*c-myc*$^{fl/fl}$;*mx-cre*$^{-}$ *or ik*$^{neo/+}$;*c-myc*$^{fl/+}$;*mb1*$^{cre/+}$). Mice were genotyped using a PCR-based analysis of tail genomic DNA (28). Primers hcre-DIR (5'-ACC TCT GAT GAA GTC AGG AAG AAC-3'), hcre-REV (5'-GGA GAT GTC CTT CAC TCT GAT TCT-3'), mb1in1 (5'-CTG CGG GTA GAA GGG GGT C-3') and mb1in2 (5'-CCT TGC GAG GTC AGG GAG CC-3') were used to amplify *mb1-cre* (hcre-DIR and hcre-REV) and *mb1-wt* alleles (mb1in1 and mb1in2). The knock-in allele (*ik*$^{neo/+}$) was identified as described (30). c-*myc*$^{fl/fl}$;*mx-cre*$^{+}$ or *c-myc*$^{fl/fl}$;*mb1*$^{cre/+}$ mice were bred with *rosa26*$^{gfp/gfp}$ "reporter" mice (31) to generate *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$;*rosa26*$^{gfp/gfp}$ or *c-myc*$^{fl/fl}$;*mb-1*$^{cre/+}$;*rosa26*$^{gfp/gfp}$ mice, respectively. The *rosa26*^{gfp} allele was genotyped as described (31).

pIpC injections. To induce *c-myc* deletion in *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ and *ik*$^{neo/+}$;*c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice, 4- to 6-week-old animals received three intraperitoneal (i.p.) injections of polyinosinic-polycytidylic acid (pIpC, Amersham) (200 μg each) at two-day intervals, and were analyzed 3 days after the last dose.

Flow cytometry analysis and cell sorting. For cell sorting or flow cytometry analysis BM B lymphocytes were purified (FACS Coulter cell sorter) and/or analyzed as (Ly6c^{-}NK1.1^{-}DX5^{-}B220^{+}c-Kit^{+}IgM^{-}) pro-B and (Ly6c^{-}NK1.1^{-}DX5^{-}B220^{+}CD25^{+}IgM^{-}) pre-B cells. Purity >97% was verified by flow cytometry re-analysis. Anti-B220 antibodies were conjugated either with PeCy7 (Bioscience), FITC or APC (Becton Coulter). Anti-IgM antibodies (Southern Biotechnologies) were conjugated either with PE or biotin. APC anti-CD19 antibody was from Becton Coulter. PE-anti-CD25, biotin-anti-CD43, PE-anti-CD117, and biotin-anti-pre-BCR antibodies were all from Pharmingen. APC-streptavidin (Pharmingen) or ECD-streptavidin (Immunotech) were used to conjugate

with biotin. FITC- or biotin–conjugated anti-Ly6.C (Becton Coulter), anti-NK1.1 and anti-DX5 (both from Pharmingen) antibodies were used in a dump channel to remove contaminating NK and dendritic cells.

BrdU labelling. BrdU incorporation was assessed 2 h after a single intravenous BrdU injection (1 mg/15 g body weight, Sigma). ($Ly6c^{-}NK1.1^{-}DX5^{-}B220^{+}c\text{-}Kit^{+}IgM^{-}$) pro-B and ($Ly6c^{-}NK1.1^{-}DX5^{-}B220^{+}CD25^{+}IgM^{-}$) pre-B cells from *mb1-fl/fl* and *mb1-fl/+* mice were sorted from BM, and BrdU incorporation measured using FITC- or PE-anti-BrdU monoclonal antibody (Becton Dickinson) or propidium iodide, following standard protocols. The same protocol for BrdU incorporation was used for sorted $B220^{+}IgM^{-}$, and immature cells from *mx-fl/fl* and *fl/fl* control mice.

Retrovirus production and transduction. Plat-E cells were seeded (2 x 10^6 cells) in 6 cm plates, 18-24 h before transfection with MIG-RI or MIG-EBF plasmids (3 μg) in the presence **of** FuGene 6 reagent (Roche Diagnostics). Retroviral supernatants were collected 48 h post-transfection and filtered through a 45 μm low protein-binding syringe filter (Pall, Life Sciences). Lin^{-} precursors were purified from total BM suspensions with Streptavidin Dynabeads (Invitrogen) by incubation with a cocktail of biotinylated antibodies to lineage markers (B220, IgM, CD4, CD8, Ter119, Gr1 and CD11b, all from Pharmingen). Cells were cultured at 10^6 cells/ml in 24-well plates in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated FBS, 1 mM L-glutamine and supplemented with recombinant murine stem cell factor (50 ng/ml), recombinant murine IL-6 (5 ng/ml) and murine leukaemia inhibitory factor (10^3 units/ml). Stimulated Lin^{-} cells were transduced by spin infection after 24 h in culture. Cells were resuspended in 1 ml fresh retroviral supernatant, supplemented with 10 μg/ml polybrene (Sigma-Aldrich) and cytokines as above. Cells were centrifuged (1136 xg, 90 min, 32ºC) and incubated (3-4 h). Medium was replaced with IMDM containing 2% heat-inactivated FBS, 1 mM L-glutamine, 1 mM penicillin/streptomycin, 0.03% w/v primatone RL (Sigma), 50 mM β-mercaptoethanol and supplemented with rSCF (10 ng/ml), rFlt3L, (10 ng/ml) and rIL-7 (10 ng/ml). Transduction efficiency was monitored by flow cytometry at 48 or 72 h post-infection.

VDJ recombination analysis. Genomic PCR amplification of immunoglobulin genes

was performed with Vh-specific primers from Fuxa *et al.* (32); DJ primers were from Ehlich *et al.* (33). GAPDH was used as loading control. PCR products were electrophoresed and the bands quantified with ImageJ software. For VDJ sequencing, PCR fragments were amplified with FastStart High Fidelity polymerase (Roche) and the V_HJ558-J_H3 band was cloned in PCRII-TOPO. Sequences were analyzed with the IGMT Junctions Analysis program.

Gene expression analysis. For qPCR analysis, 2.5 μl cDNA (ten-fold dilution series) was mixed with primers and SYBR Green PCR master mix (Becton Dickinson). All oligonucleotides were designed to yield 70-130 bp PCR fragments. Oligonucleotides for *c-myc* and *β-actin* were as described (34). Primers for *cd19* were CD19 sense (s) (5'-AGTACGGGAATGTGCTCTCC-3') and antisense (as) (5'-GGACT TGAATGCGTGGATTT-3'), E2A-s (5'-ATACAGCGAAGGTGCCCACT-3') and E2A-as (5'-CTCAAGGTGCCAACACTGGT-3') for *tcfe2a,* EBF1-s (5'-CTATGTGCGCCTCAT CGACT-3') and as (5'-CATGATCTCGTGTGTGAGCAA-3') for *ebf-1*, Flt3-s (5'-CAGCCG CACTTTGATTTACA-3') and as (5'-GGCTTCGCTCTGAATATGGA-3') for *flt3*, Ikaros-s (5'-TTGTGGCCGGAGCTATAAAC-3') and as (5'-TGCCATCTCGTTGTGGTTAG-3') for *ikaros*, Lef-1-s (5'-TCAGTCATCCCGAAGAGGAG-3') and as (5'-CTCTGGCCTTGTCGT GGTAG-3') for *lef-1*, Pu-1-s (5'-GGGATCTGACCAACTGGAG-3') and as (5'-GCTGCCC ACGAAGGAGTAGTA-3') for *spfi1*, and Rag1-s (5'-GTTGCTATCTCTGTGGCATCG-3') and as (5'-AATTTCATCGGGTGCAGAAC-3') for *rag1*. For the remainder of the genes, Pre-designed Applied Biosystems Micro Fluidic cards were used. Each gene was analyzed in triplicate. cDNA samples and reagents were run on an ABI Prism 7900HT. Data were analyzed with SDS2.2 sequence detection systems.

Luciferase activity assay. The murine *ebf-1* α-promoter was amplified by PCR using the following primers: Ebf1-α 5: 5'-TAAGAGCGCGGAACTGTCC-3' and Ebf1-α 3: 5'-GCTGAAGAATCTGCCAGAAGTT-3', cloned into the *Eco*RI site of the Topo 2.1 vector (Invitrogen) and subcloned into the *Nhe*I-*Bgl*II site of the pGL3-Control vector (Promega) upstream of the luciferase gene to generate the vector pGL3-Ebf1α. All constructs were sequenced. For luciferase assays, HEK-293T cells were cultured in 24-well plates and cotransfected with 500 ng pGL3-Ebf1α or pGL3 control vector and

increasing amounts of pRV-IRES-GFP-c-Myc expression vector. *Renilla* luciferase activity was used to normalize. At 48h post-transfection, firefly and *Renilla* luciferase activity were measured using the Dual-Luciferase Reporter Assay (Promega).

ChIP assays. Experiments were performed following the protocol of the ChIP assay kit (Active Motif). L1-2 cells were crosslinked with formaldehyde (1% final concentration) and incubated (room temperature, 20 min). Rabbit polyclonal anti-c-Myc N262 antibody (sc-764, Santa Cruz) or pre-immune serum was used to precipitate chromatin from 2 x 10^6 cells. Immunoprecipitated DNA and input samples were analyzed with SYBR Green RT-PCR Kit (Applied) and percent enrichment relative to the amount of input chromatin was determined as $2^{(Ct\ input - Ct\ antibody)}$. Primers flanking E-box5 were: EB5-FW (5'-CCTCAGCTCGTTCTGAGAGG-3') and EB5-REV(5'-ACTCGCAGGAGGTAGAG AACG-3).

EMSA. Assays were performed with labeled double-stranded (ds) oligonucleotides encompassing E-boxes from the *dhfr* and *ebf-1* α promoters. pcDNA3-c-Myc and pcDNA3-Max were *in vitro* translated (IVT) using TNT-coupled reticulocyte lysate systems (Promega). Binding reactions between IVT proteins and labeled probes (1 ng) were performed as described (35), except that 0.25x TBE was used. Unlabeled oligonucleotide competitors (100 ng) and either 1 μg anti-c-MYC sc-764x or 1 μg anti-c-MYB sc-517x antibodies (both from Santa Cruz) were used. Ds oligonucleotides used: DHFR-I-wt (5'-GGCGCGACACCCACG TGCCCT-3' and 5'-AGAGAGGGCACGTGGGTGTCG-3'), EB5-wt (5'-GGTCCTACCCA CGTTGACTGCAGT-3' and 5'GAGACTGCAGTCAACGTGGGTAGGA-3'), EB5-mut (5'-GGTCCTACCCTTGCTGACTGCAGT-3' and 5'-GAGACTGCAGTCAGCAAGGGTAGG

A-3'), EB505-wt (5'-CGTTTCCTCACCTGTACAATGGGAGTGG-3' and 5'-GTCCACTC

CCATTGTACAGGTGAGGAAA-3'), EB505-mut (5'-CGTTTCCTCTCTTATACAATGG

GAGTGG-3' and 5'-GTCCACTCCCATTGTATAAGAGAGGAAA-3'). Complementary oligonucleotides were mixed at an equimolar ratio in 10 mM Tris (pH 7.5)-50 mM NaCl, heated to 65°C, and annealed by slow cooling to room temperature. Double-stranded oligonucleotides (100 ng) were labeled by a Klenow fill-in reaction.

RESULTS

c-Myc is necessary for B lymphocyte differentiation

To study the role of c-Myc in B cell differentiation, we conditionally inactivated *c-myc* gene in developing B lymphocytes by breeding the *c-myc$^{fl/fl}$* (*fl/fl*) (21) conditional mouse with *mb-1$^{cre/+}$* knock-in (29), *mx-cre* transgenic (36), and *rosa26$^{gfp/gfp}$* "reporter" mice (31). In *c-myc$^{fl/fl}$;mb1$^{cre/+}$* mice (*mb1-fl/fl*), the *mb1-cre* allele is expressed from the earliest stage of B lymphocyte differentiation (29). In *c-myc$^{fl/fl}$;mx-cre^{+}* mice (*mx-fl/fl*), Cre recombinase is induced after injection of interferon or polyinosinic-polycytidylic acid (pIpC) and efficiently deletes *c-myc* in bone marrow (BM) (28). In addition, in *rosa26$^{gfp/gfp}$;c-myc$^{fl/fl}$;mb1$^{cre/+}$* mice (*gfp-mb1-fl/fl*) and in *rosa26$^{gfp/gfp}$;c-myc$^{fl/fl}$;mx-cre^{+}* mice (*gfp-mx-fl/fl*), the *rosa26$^{gfp/gfp}$* allele expresses green fluorescent protein (GFP) following activation of the Cre recombinase (31) (supplemental Fig. S1*A*).

To determine whether *c-myc* inactivation affects B cell differentiation, we used flow cytometry to analyze the B cell populations in the BM and spleen of *mb1-fl/fl* mice (37) (38). Deletion of the *c-myc* gene at early differentiation stages led to a developmental defect at the pro- to pre-B cell transition in BM of *mb1-fl/fl* and *gfp-mb1-fl/fl* mice (Fig. 1, *A* and *B*; supplemental Fig. S1*B*). Analysis of Hardy fractions in these mice also revealed a developmental defect and a decrease in absolute numbers in Fraction B, C and C´ (Large pre-B cells) consistent with the time in which deletion of *c-myc* occurs (Fig. 1, *C* and *D*). Similar results were obtained when CD19 was used as a B cell marker (supplemental Fig. S2, *A-D*). The absolute number of B lymphocytes (B220^{+}) in *mb1-fl/fl* mouse BM was four-fold lower than those of controls (0.4 x 10^{6} vs 1.8 x 10^{6}) (Fig. 1*E*). *mb1-fl/fl* spleens showed a 34-fold decrease (0.2 x 10^{6} vs 8.1 x 10^{6}) in the number of mature B lymphocytes (B220^{+}IgM^{+}) compared to controls (Fig. 1*E*). Genomic PCR analysis of these cells confirmed *c-myc* deletion (supplemental Fig. S1, *D* and *E*). *In vitro* cultures of *gfp-mb1-fl/fl* mouse BM cells did not generate IgM^{+} B lymphocytes, suggesting that the absence of c-Myc in *mb1-fl/fl* and *gfp-mb1-fl/fl* mouse BM prevents generation of mature B cells in spleen (supplemental Fig. S3). To test the apoptotic status of pro- and pre-B cells in *mb1-fl/fl* mouse BM, we monitored

apoptosis by flow cytometry using annexin-V, and found a two-fold (2.8% vs 6.1%) in pro-B cells and a twenty-five-fold increase in pre-B cells (0.9% vs 23.1%) from *mb1-fl/fl* mice compared to controls (Fig. 2*A*). c-Myc appears to be necessary for pre-B cell survival in *mb1-fl/fl* mice, and thus to be required from early stages of B cell differentiation.

To study the c-Myc requirement at later stages of B cell development, we injected pIpC to induce *c-myc* deletion in *mx-fl/fl* mouse BM. Analysis of Hardy fractions showed a decrease in the absolute numbers from fraction A to fraction E (Fig. 1*F* and *G* and supplemental Fig. 2*E*, *F* and *G*). Fraction F seems to be less affected consistent with our previous results in mature B cells (21) (Fig. 1, *F* and *G*). Annexin-V staining showed an increase (35.8% vs 14.1%) in the relative numbers of apoptotic pro- and pre-B ($B220^{+}IgM^{-}$) and immature B cells ($B220^{+}IgM^{+}$) (77.9% vs 27.6%) in *mx-fl/fl* mouse BM compared to controls (Fig. 2*B*). Recirculating mature ($B220^{++}IgM^{+}$) B lymphocytes in *mx-fl/fl* mouse BM survived in the absence of c-Myc (26.6% vs 31.5%), consistent with results for $c\text{-}myc^{fl/fl};cd19^{cre/+}$ mice (21), indicating that *c-myc* is dispensable for mature B lymphocyte maintenance (Fig. 1*F* and Fig. 2*B*). The lack of early hematopoietic precursors (28) could also contribute to the decrease in the number of recirculating mature B cells in *mx-fl/fl* mouse BM (not shown). These results indicate that c-Myc is necessary for the generation of pro- and pre-B cells and for maintenance of immature B lymphocytes.

Cell proliferation in developing c-Myc-deficient B lymphocytes

c-Myc promotes proliferation in many cell types, including B lymphocytes, by regulating cell cycle genes (19). Pro- to pre-B cell transition is also characterized by cell expansion (39). To determine whether c-Myc inactivation affected cell proliferation, we monitored *in vivo* BrdU incorporation in *mb1-fl/fl* and pIpC-injected *mx-fl/fl* mice. Sorted pro- and pre-B lymphocytes from *mb1-fl/fl* mice still retain some capacity to proliferate compared to controls (54.5% vs 25.4% in pro-B and 51.5% vs 6.1% in pre-B $BrdU^{+}$ cells) by flow cytometry analysis (Fig. 3*A*). *mx-fl/fl* mouse pro- and pre-B cells showed a similar proliferative capacity (Fig. 3*B*). We did not observe significant

differences in proliferation in immature lymphocytes from *mx-fl/fl* mice (Fig. 3*B*). We concluded that developing B lymphocytes have a reduced capacity to proliferate in the absence of c-Myc *in vivo*.

Reduced levels of V(D)J recombination in c-Myc deficient B lymphocytes

Sequential rearrangement of the V(D)J gene segments that encode the B cell receptor is linked intrinsically to B lymphocyte differentiation (2). To determine whether the developmental defect in c-Myc-deficient B lymphocytes was characterized by a lack of or impaired immunoglobulin gene recombination, we analyzed V(D)J rearrangement by genomic PCR in sorted pro-B and pre-B cells. We did not observe significant differences in Dh-to-Jh, $Vh_{proximal}$-, and Vh_{distal}-to-DhJh rearrangements in purified pro-B cells from *mb1-fl/fl* mouse BM compared to control cells (Fig. 3*C*, top panel, and Fig. 3*D*, $p>0.05$). In contrast, purified pre-B cells from mutant mice showed a slight decrease in $Vh_{proximal}$- (1.9-fold) and Vh_{distal}-to-DhJh (2.4-fold) rearrangements compared to control mice (Fig. 3*C*, bottom panel, and Fig. 3*D*). These differences between pro- and pre-B likely reflect the time in which *c-myc* deletion occurs. Pro-B cells are undergoing *c-myc* deletion and pre-B cells have completed it. To see whether these recombination events were normal we sequenced some V(D)J rearrangements from *mb1-fl/fl* and *mb1-fl/+* mouse B cells and observed no apparent differences between both populations (supplemental Table S1).

Gene expression in c-Myc-deficient B lymphocytes

To define the molecular mechanism by which c-Myc acts on B lymphocyte differentiation, we analyzed gene expression of key transcription factors involved in this process. Quantitative PCR (qPCR) showed that *tcfe2a*, *ebf-1, ikaros* and *pax-5* expression was slightly downregulated in sorted c-Myc-deficient pro-B cells and this effect was more dramatic in pre-B cells. This is likely due to the timing in which *c-myc* deletion occurs in *mb-1-cre* mice. (Fig. 4*A*). Interestingly, *tcfe2a*$^{-/-}$, *ebf-1*$^{-/-}$ and *pax-5*$^{-/-}$ mice have a block at early stages of B cell development, as well as impaired V(D)J

recombination (4, 6, 9). EBF-1 shares some target genes with Pax-5 and transcriptionally regulates its expression providing B cell identity (7). Expression of Pax-5 target genes such as *cd19* (11) was reduced and that of the repressed target gene *flt3* (40) was increased in pro-B and pre-B cells from *mb1-fl/fl* mice compared to controls (Fig. 4*A*). Flow cytometry showed fewer pro- and pre-B cells with surface expression of CD19 (7.2% vs 68.4%) or pre-BCR (0.1% vs. 0.3%) in *mb1-fl/fl* mouse BM compared to controls (Fig. 4, *B* and *C*). Interestingly, *flt3* gene expression was highly increased in c-Myc deficient pre-B (36-fold) compared to control cells, higher than described for *pax-5*$^{-/+}$ B cells (14). This probably reflects the need for additional factors under the control of c-Myc, other than Pax-5, required for normal regulation of *flt3* promoter. In contrast, *cd19* promoter, under the tight control of Pax-5 is more sensitive to small variations of gene expression of this transcription factor (Fig. 4*C*).

Consistent with these results, gene expression of Pax-5 target genes *blnk* (12)*, cd79a, cd79b, n-myc* (41) was reduced in B220^{+}IgM^{-} cells (pro- and pre-B) from *mb1-fl/fl* mice (supplemental Fig. S4*A*). Similarly, pro- and pre-B cells from *gfp-mx-fl/fl* mice showed reduced *cd79b, cd79a* and *pax-5* expression (supplemental Fig. S4*B*). To test whether decreased *pax-5* expression promoted transdifferentiation of c-Myc-deficient B cells into T cells (42), we cultured pro- and pre- B cells with the OP9-DL-1 cell line (43). Genomic PCR analysis indicated no TCR recombination in cultures of c-Myc-deficient B cells from *gfp-mb1-fl/fl* mouse BM (supplemental Fig. S5).

EBF-1 is a transcriptional target of c-Myc

ebf-1 gene expression is controlled by two promoters, α and β , which are differentially regulated in B cells (44). We identified two conserved c-Myc-binding sites (E-boxes) in human and mouse, upstream of or within the *ebf-1* α-promoter. In reporter assays on 293T fibroblasts, a 1.5 Kb genomic region containing the *ebf-1* β-promoter did not activate the *luciferase* gene in a c-Myc dose-dependent manner (supplemental Fig. S5, *A* and *B*). Mutant deletion analysis identified a 0.9 Kb region of the *ebf-1* α-promoter that activated the *luciferase* reporter in a c-Myc dose-dependent manner in fibroblasts (2.5-fold) and in the L1-2 B cell line (2-fold) (Fig. 5, *A*-*C*). Luciferase assays showed

that site-directed mutagenesis of the E-box5 (EBΔ5), located 200 bp upstream of the transcription start site, completely abolished basal promoter activity and c-Myc-dependent *ebf-1* transactivation in both cell lines (Fig. 5, *A*-*C*). c-Myc-dependent transactivation was not observed with genomic regions containing the *pax-5* or *tcfe2a* promoters (supplemental Fig. S5, *C* and *D*).

To determine whether c-Myc binds to a genomic region containing E-box5, we performed chromatin immunoprecipitation assays (ChIP) in L1-2 cells (45). Using specific primers that flank E-box5, we observed a 10-fold enrichment by PCR of the DNA fragments immunoprecipitated with a c-Myc-specific antibody compared to pre-immune serum (Fig. 5*D*). We used EMSA assays to determine whether c-Myc bound specifically to this E-box5; c-Myc bound to oligonucleotides containing E-box5 from the *ebf-1* locus. Mutated E-box5 or E-box4 did not compete for c-Myc binding with unmutated E-box5, as determined using anti-c-Myc antibody (Fig. 5*E*, top). c-Myc binds to an E-box located in a region 5´ of the *dhfr* gene (46). We observed that E-box5 from *ebf-1* competed for c-Myc binding with oligonucleotides containing *dhfr* E-box. Mutated *ebf-1* E-box5 or E-box4 did not compete with the *dhfr* E-box (Fig. 5*E*, bottom). Together these data show that c-Myc directly regulates *ebf-1* transcription by binding to the E-box5 in the *ebf-1* α-promoter.

In vitro rescue of B cell differentiation in c-Myc-deficient B lymphocytes

To determine whether restoration of EBF-1 expression in c-Myc-deficient B lymphocytes promotes B cell differentiation, we cultured BM progenitors (Lin$^-$) from *mb1-fl/fl* and *mb1-fl/+* control mice and infected them with a retrovirus expressing EBF-1-GFP or a GFP-control vector. After six days in culture with IL-7, the BM progenitors from *mb1-fl/fl* mice infected with EBF1-expressing retrovirus generated c-Myc-deficient B220$^+$CD19$^+$GFP$^+$ cells (Fig. 6*A*). We did not observe surface expression of IgM in c-Myc-deficient B220$^+$CD19$^+$GFP$^+$ infected with EBF1-expressing retrovirus (not shown). To see whether EBF-1-induced differentiation in c-Myc deficient B cells affected V(D)J recombination we performed genomic PCR on these cells. Genomic DNA was isolated from either sorted B220$^+$CD19$^+$GFP$^+$ or B220$^+$CD19$^-$GFP$^+$ cells infected with

either EBF-1 or GFP control retrovirus from *mb1-fl/fl* and *mb1-fl/+* control mice. $B220^+CD19^+GFP^+$ population was not generated from *mb1-fl/fl* mouse when infected with control retrovirus (Fig. 6A). We observed an increase in Dh-to-Jh rearrangements in EBF-1-infected $B220^+CD19^+GFP^+$cells compared to $B220^+CD19^-GFP^+$ infected with control retrovirus (Fig. 6*B*). Vh- to DhJh rearrangements were hardly detected in both populations infected with either retrovirus (Fig. 6*B*). We concluded from these experiments that EBF-1 promoted B cell differentiation in c-Myc deficient B cells by inducing CD19 expression and contributing to DJ rearrangements.

To see whether rescue by EBF-1 of B lymphocyte differentiation in c-Myc deficient B cells affected cell proliferation $B220^+CD19^+$ infected cells were stained with propidium iodide from *mb1-fl/fl* and *mb1-fl/+* control mice. We observed that expression of EBF-1 did not restore the normal capacity to proliferate (39.4% vs 11.8 %) in c-Myc deficient B cells (Fig. 6*C*).

Pax-5 is transcriptionally regulated by EBF-1 and activates B cell-specific genes such as *cd19* (11, 44), conferring B cell identity on these cells (7). To test whether ectopic expression of EBF-1 was accompanied by activation of *pax-5* expression, we performed qPCR in sorted $B220^+CD19^+GFP^+$ cells. c-Myc-deficient $B220^+CD19^+GFP^+$ cells expressed higher *pax-5* levels than did control retrovirus-infected c-Myc-deficient $B220^+CD19^-GFP^+$ cells from the same mice. We did not observe changes in gene expression in *n-myc* and *tcfe2a* (Fig. 6*D*). We concluded that ectopic expression EBF-1 promotes B cell differentiation by inducing CD19 expression in c-Myc deficient B lymphocytes.

To test whether Pax-5 expression alone contributed to the rescue of B lymphocyte differentiation in c-Myc-deficient B lymphocytes, we bred *mx-fl/fl* with $ik^{neo/+}$ mice (30) to generate $ik^{neo/+}$;$c\text{-}myc^{fl/fl}$;$mx\text{-}cre^+$ mice (*ik-mx-fl/fl*). $ik^{neo/+}$ mice express *pax-5* from the endogenous *ikaros* promoter upon deletion by the Cre recombinase of a stop codon flanked by loxp sites. In *ik-mx-fl/fl* mice, pIpC injection leads to Cre recombinase expression and deletion of *c-myc* and activation of *pax-5* expression from the endogenous *ikaros* promoter. Attempts to rescue B cell differentiation by expressing *pax-5* in *ik-mb-1-fl/fl* mice were unsuccessful probably due to the low levels

of *pax-5* expression in these mice (supplemental Fig. S7, *A*-*C* and Fig. S8). Flow cytometry analysis of B cell populations in the BM of pIpC-injected *ik-mx-fl/fl* mice showed no significant differences in the number of c-Myc deficient pro- and pre-B cells and a five-fold increase in immature B lymphocytes (0.5 x 10^6 vs 0.1 x 10^6) compared to *mx-fl/fl* mice (Fig. 7, *A*, *B* and *C*). We also observed an increase in the number of CD19-expressing pro- and pre-B cells in *ik-mx-fl/fl* mice (73.1% vs 42.5%) (Fig. 7*A*). We concluded that Pax-5 contributed to promote B cell differentiation in c-Myc deficient B lymphocytes.

DISCUSSION

Since the discovery of c-Myc, an extensive scientific literature has addressed its function in various experimental settings (16). The prominent role of c-Myc in the cell cycle and in apoptosis has been the focus of many reports using various cell types, including B lymphocytes. The specific function of this gene in B lymphocyte differentiation nonetheless remains poorly understood, probably due to the lack of mouse models suitable for its study. Here we addressed the role of c-Myc in B cell differentiation *in vivo*, and found that c-Myc regulates this process in part by conferring identity to early B lymphocyte precursors.

The generation of *mb1-fl/fl* and *mx-fl/fl* mouse models allowed us to define the requirements for c-Myc in B lymphocytes at distinct developmental stages. Conditional inactivation of *c-myc* in *mb1-fl/fl* mice showed that this gene is required at least from pre-B to immature B cell stages; we observed a reduction in pre-B cells and increased apoptosis in these cells (Fig. 1 and 2). Fewer pro-B cells are affected than pre-B cells, probably due to the time at which *c-myc* deletion occurs in these mice. The reduced number of cells that express GFP in *gfp-mb1-fl/fl* mouse BM probably reflects increased apoptosis in c-Myc-deficient B lymphocytes as well as the accessibility of both loci to Cre recombinase (47). A similar block during transition from the pro-B cell to the pre-B cell has been described for the *mb1-cre*-mediated deletion of *c-myb*, a known gene regulating *c-myc* (48). Our results are in agreement and provide evidence of a more prominent role of c-Myc in collaboration with c-Myb in the regulation of these processes via EBF-1.

The increased apoptosis observed at all developmental stages except in mature B cells (21), and the inability of *mx-fl/fl* (34) and *mb1-fl/fl* mice to generate B220$^+$IgM$^+$ cells *in vivo* and *in vitro* (Fig. 2 and supplemental Fig. S3) show the need for c-Myc in B cell generation and maintenance during differentiation. The c-Myc requirement in hematopoietic stem cell differentiation (34, 49) probably contributes to the decreased number of early B cell precursors in *mx-fl/fl* mouse BM.

The role of c-Myc in regulating the G1-S transition of the cell cycle in different cell types has been widely studied (19). At early stages, we observed that B cells lacking

c-Myc retain limited proliferative capacity in both *mb1-fl/fl* and *mx-fl/fl* mice (Fig. 3, *A* and *B*). This ability to proliferate in the absence of c-Myc has been reported for other cell types (28, 49, 50). It is possible that cells are already cycling and that *c-myc* is deleted at stages when the protein is less critical to continue through the cell cycle. This might be more relevant at the transition between pro- and pre-B cell stages, when extensive expansion occurs (Fig. 3*A* and *B*). Alternatively, this could reflect distinct c-Myc requirements for cell proliferation, depending on the developmental stage.

We did not observe c-Myc-dependent transcriptional regulation of *tcfe2a* or *pax-5* promoters in luciferase reporter assays (supplemental Fig. S6). The reduced *tcfe2a* gene expression in c-Myc-deficient B lymphocytes nonetheless suggests an indirect effect of c-Myc on *tcfe2a* regulation. It remains to be determined whether E2A expression in c-Myc-deficient B lymphocytes is sufficient to promote B lymphocyte differentiation. We identified *ebf1* as a previously unreported c-Myc target gene. The contribution of the *ebf1* alpha promoter to the total level *ebf1* transcripts is little compared to the beta promoter. However, we believe that this contribution is essential at early stages of B cell differentiation due to the complex regulation of *ebf-1* expression as previously described (44). The activity of the *ebf-1* alpha promoter will induce expression of *pax-5* which in turn will activate the *ebf-1* beta promoter. **The activity of the *ebf-1* alpha promoter would induce expression of *pax-5* which in turn could activate the *ebf-1* beta promoter.** In c-Myc deficient B cells **activation** of *ebf-1* alpha promoter will be compromised, and therefore the total amounts of EBF-1 mRNA will be dramatically reduced.

Our results indicate that c-Myc regulates cell proliferation and survival in developing B lymphocytes; c-Myc function is thus not restricted to the regulation of *ebf-1* expression in B cell differentiation.

The observation that c-Myc-deficient pre-B cells undergo a slight reduction but normal V(D)J recombination despite decreased *tcfe2a* (4, 5), *ebf-1* (6), and *pax-5* (10, 51) expression probably reflects cell pool heterogeneity while undergoing *c-myc* deletion. This became more evident when we compared c-Myc-deficient and *pax-5*$^{-/-}$ B cells.

Despite reduced *pax-5* expression in c-Myc-deficient B lymphocytes, we observed minimal differences in the levels of $Vh_{proximal}$- and Vh_{distal}-to-DhJh recombination (10). In c-Myc deficient B cells, enforced expression of EBF-1 induces surface expression of CD19 and slightly increases the levels of D to J recombination in these cells (Fig. 6*B*). These results might reflect a broad rather than a specific effect of c-Myc deficiency on V(D)J machinery (51).

Unlike *pax-5*$^{-/-}$ B cells (52), c-Myc-deficient B lymphocytes were unable to differentiate to other cell lineages *in vivo* and *in vitro* (supplemental Fig. S5 and not shown). Although we did not detect $B220^{-}GFP^{+}$ cells in the thymus, bone marrow or spleen of *gfp-mb1-fl/fl* mice (not shown), it is nonetheless possible that lack of c-Myc confers on B lymphocytes the ability to differentiate to other cell lineages. To test this, the increased viability of c-Myc-deficient B cells is essential. Our attempts to rescue c-Myc-deficient B lymphocytes from apoptosis by breeding *mx-fl/fl* with E_{μ}-*bcl-2* transgenic mice (53) were unsuccessful (not shown).

The capacity of EBF-1 to induce *pax-5* gene expression (41, 54, 55) and to activate the B cell transcription program could explain its ability to promote B cell differentiation in c-Myc-deficient cells *in vitro,* despite the large number of genes regulated by c-Myc (Fig. 6). *pax-5* expression in *ik-mb-1-fl/fl* mice nonetheless did not rescue B cell differentiation (supplemental Fig. S6). This might be attributed to the brief time frame available for expression of normal Pax-5 levels before cell death following c-Myc deletion (supplemental Fig. S7). In contrast, Pax-5 expression in *ik-mx-fl/fl* mice contributed to a significant increase in the number of c-Myc-deficient immature B cells and to cell surface expression of the Pax-5 target CD19 (Fig. 7*A*). Pro- and pre-B cell number did not increase significantly in *ik-mx-fl/fl* mice as observed by Souabni et al. (30) (Fig. 7*B*). In our experimental model, developing B lymphocytes show increase cell death upon deletion of *c-myc* (Fig. 2). This effect probably makes more difficult to increase B cell numbers in our system than in Souabni et al. (30) where Pax-5 is overexpressed in a normal background. Our system does not allow to control when *c-myc* deletion and/or *pax-5* expression occur with respect to each other. Moreover, c-Myc affects B lymphocytes depending on the differentiation stage (21, 22). Altogether, these effects might account for the differences in cell number in immature B

lymphocytes in *ik-mx-fl/fl* mice.

Our results identified *ebf-1* as an unreported c-Myc target gene, and illustrate a novel c-Myc function in the regulation of B lymphocyte differentiation. Through *ebf-1* activation, c-Myc regulates differentiation by promoting B cell identity. These data show that c-Myc not only regulates *ebf-1* but also affects multiple biological functions during B lymphocyte differentiation such us cell survival or proliferation. The capacity of c-Myc to regulate these functions has been widely studied (16, 19).

Finally, this study places c-Myc within the context of transcription factors essential for B lymphocyte differentiation by linking this transcription factor to the EBF-1-Pax-5 pathway. Based on these data, a model emerges for transcriptional regulation of B lymphocyte differentiation in which c-Myc acts by regulating B or T cell-specific transcription factors. This model postulates a requirement for one or more additional factor(s) to allow c-Myc to discriminate between B and T cell lineages.

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DISCLOSURES

The authors have no financial conflict of interest.

FIGURE LEGENDS

FIGURE 1. c-Myc is necessary for B lymphocyte differentiation. *A*, *B*, B lymphocyte differentiation is blocked at the pre-B cell stage in *mb1-fl/fl* mouse BM. Single-cell suspensions were prepared from *mb1-fl/fl* and *mb1-fl/+* mice, stained and analyzed by flow cytometry (see methods). Cells were defined as ($Ly6c^{-}NK1.1^{-}DX5^{-}B220^{+}c\text{-}Kit^{+}IgM^{-}$) pro-B and ($Ly6c^{-}NK1.1^{-}DX5^{-}B220^{+}CD25^{+}IgM^{-}$) pre-B cells. *C*, Flow cytometry analysis of Hardy Fractions in BM of *mb1-fl/fl* and *mb1-fl/+* control mice. $B220^{+}CD43^{+}$ and $B220^{+}CD43^{-}$ gates and NK and dendritic cells discrimination were as 1A. *D*, *E*, Absolute numbers of B lymphocytes in *mb1-fl/fl* and control mouse BM (*n* =7) and spleen (*n*=3). *F*, Flow cytometry analysis of B lymphocytes from pIpC-injected *mx-fl/fl* and *fl/fl* mouse BM. $mb1\text{-}fl/^{+}$ = $c\text{-}myc^{fl/+};mb\text{-}1^{cre/+}$. *G*, Flow cytometry analysis of Hardy Fractions in BM of *mx-fl/fl* mice. Data represent one of ≥3 independent experiments. *p* values are ***$p<0.001$, **$p<0.01$, *$p<0.05$.

FIGURE 2. Increased apoptosis in developing c-Myc deficient B lymphocytes. *A*, Apoptosis in pro- and pre-B cells in *mb1-fl/fl* mice. Cells were antibody- and annexin-V-stained and gated as in Fig. 1*B*. *B*, Pro-, pre- and immature B lymphocytes die by apoptosis following *c-myc* deletion in *mx-fl/fl* mice. BM cells were stained as in Fig. 1*F*, and B cell populations analyzed by flow cytometry using gates as in Fig. 1 *F*. Experiments representative of three independent experiments.

FIGURE 3. Cell proliferation and V(D)J recombination of c-Myc-deficient B lymphocytes. *A*, Decreased proliferation of sorted pro-B and pre-B cells from *mb1-fl/fl*. Cells were stained as in 1*B* and BrdU incorporation was measured by flow cytometry in ($Ly6c^{-}NK1.1^{-}DX5^{-}B220^{+}c\text{-}Kit^{+}IgM^{-}$) pro-B and ($Ly6c^{-}NK1.1^{-}DX5^{-}B220^{+}CD25^{+}IgM^{-}$) pre-B cells in the BM. *B*, Pro- and pre-B cells from *mx-fl/fl* mouse BM. Mice were BrdU-injected 2 h before analysis; *n*=3 for *mb1-fl/fl* and *n*=5 for *mx-fl/fl*. *C*, V(D)J recombination analysis in sorted pro-B and pre-B cells

from *mb1-fl/fl* or control mice. Genomic PCR from sorted BM pro- and pre-B cells defined as in 1*B* was performed using specific primers to detect D to J_H or V_H to DJ_H rearrangements (see Methods). Three mice of each genotype were analyzed; DNA from *rag-1*$^{-/-}$ was included as negative control. *D,* VDJ rearrangement levels were quantified by measuring the fluorescent intensity of the amplified PCR fragments in the agarose gel using ImageJ software. Fold IntDen indicates the integrated intensity average ratio of *mb1-fl/fl* vs control samples, including the three 5-fold dilutions of three different mice from each genotype, after GAPDH normalization. NS, not significant. *p* values are ***p<0.001, **p<0.01, *p<0.05

FIGURE 4. Gene expression in c-Myc deficient B lymphocytes. *A*, qPCR of sorted pro-B and pre-B cells from *mb1-fl/fl* and *mb1-fl/+* control mouse BM. Each panel shows an independent experiment (mean ± SD for 3 mutant and 3 control mice); numbers indicate the *x*-fold change ($2^{-\Delta Ct}$). *B*, *C*, Flow cytometry analysis of pre-BCR and CD19 surface expression on *mb1-fl/fl* and *mb1-fl/+* mouse BM B lymphocytes. *c-myc* transgenic (*Eμ-c-myc*) and *rag-1*$^{-/-}$ mice were included for comparison. Experiment representative of at least three independent experiments.

FIGURE 5. c-Myc transcriptionally regulates *ebf-1*. *A*, Luciferase reporter constructs. *B*, *C*, c-Myc dose-dependent activation of *ebf-1* in HEK293T and L1-2 cells (B cells) in transient transfection assays. Luciferase activity was normalized with *Renilla* activity (relative luciferase units, RLU). Mean ± SD for three replicates in one representative experiment. *p* values are ***p<0.001 and *p<0.05. *D*, ChIP assays in L1-2 B cells. Immunoprecipitation was performed with anti-c-Myc antibody (N262) or pre-immune serum (control). Data show the mean of three independent experiments. *p<0.05. *E*, EMSA assays. *In vitro*-translated c-Myc and Max proteins were incubated with oligonucleotides containing E-box5 from *ebf-1* (top) or E-box from *dhfr* (bottom) and the indicated competitor oligonucleotides. Lane 1, negative

control without c-Myc and Max. Lane 2, no competitor. Lane 9, negative control with anti-c-Myb antibody. Arrow indicates the shift of c-Myc-Max oligonucleotide complexes. All experiments are representative of at least three independent assays.

FIGURE 6. Ectopic expression of EBF-1 rescues B lymphocyte differentiation in c-Myc-deficient B lymphocytes. *A*, Lin^- cells from *mb1-fl/fl* and *mb1-fl/+* mouse BM were isolated and infected with *ebf-1-gfp*-expressing or *gfp* retrovirus. Cells were harvested after 6 days, antibody-stained and analyzed by flow cytometry. Experiment representative of at least three independent assays. *B*, V(D)J recombination in c-Myc deficient B lymphocytes ectopically expressing EBF-1. Genomic PCR was performed on DNA from sorted $B220^+CD19^+GFP^+$ or $B220^+CD19^-GFP^+$ cells infected with EBF-1 or GFP control retrovirus from a pool of four *mb1-fl/fl* and three *mb1-fl/+* control mice. *C*, Cell cycle analysis of sorted $B220^+CD19^+GFP^+$ infected with *ebf-1*-expressing retrovirus as in 6*A*. Cells were sorted and stained with propidium iodide. Experiment representative of two independent experiments. *D*, Gene expression of *pax-5, ebf-1, tcfe2a* and *n-myc* in *ebf-1*-infected cells. Cells were infected as in 6*A*, sorted and analyzed by qPCR. A pool of three mice of each genotype was used.

FIGURE 7. Pax-5 expression contributes to differentiation of c-Myc-deficient B lymphocytes. *A*, Flow cytometry analysis of *ik-mx-fl/fl* and control mouse BM. *B*, Absolute numbers of B cell subpopulations in BM of mice in *A*. $^{**}p<0.01$, *ik-mx-fl/fl*, $n = 5$; *fl/fl*, $n = 6$; *mx-fl/fl*, $n = 4$. *C*, *c-myc* deletion in sorted populations from mice of the indicated genotypes. Experiment representative of two independent experiments.

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