Fra-2 regulates B cell development by enhancing IRF4 and Foxo1 transcription

Kenia Ubieta,[1,4] Mireia Garcia,[1] Bettina Grötsch,[1] Steffen Uebe,[2] Georg F. Weber,[4] Merle Stein,[3] Arif Ekici,[2] Georg Schett,[1] Dirk Mielenz,[3]* and Aline Bozec[1]*

[1]Department of Internal Medicine 3 – Rheumatology and Immunology, Universitätsklinikum Erlangen, [2]Institute of Human Genetics, [3]Division of Molecular Immunology, Department of Internal Medicine 3 – Rheumatology and Immunology, Nikolaus-Fiebiger-Center, and [4]Department of Surgery, Friedrich-Alexander-University Erlangen-Nuremberg, Erlangen, Germany

The role of AP-1 transcription factors in early B cell development and function is still incompletely characterized. Here we address the role of Fra-2 in B cell differentiation. Deletion of Fra-2 leads to impaired B cell proliferation in the bone marrow. In addition, IL-7–stimulated pro–B cell cultures revealed a reduced differentiation from large pre–B cells to small B cells and immature B cells. Gene profiling and chromatin immunoprecipitation sequencing analyses unraveled a transcriptional reduction of the transcription factors *Foxo1*, *Irf4*, *Ikaros*, and *Aiolos* in Fra-2–deficient B cells. Moreover, expression of *IL7Rα* and *Rag 1/2*, downstream targets of Irf4 and Foxo1, were also reduced in the absence of Fra-2. Pro–B cell proliferation and small pre–B cell differentiation were fully rescued by expression of *Foxo1* and *Irf4* in Fra-2–deficient pro–B cells. Hence, Fra-2 is a key upstream regulator of *Foxo1* and *Irf4* expression and influences proliferation and differentiation of B cells at multiple stages.

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INTRODUCTION

B lymphocyte development is initiated in the bone marrow. Common lymphoid progenitor cells require the combinatorial activity of multiple transcription factors in a complex gene-regulatory network (Nutt and Kee, 2007). Ikaros and PU.1 are indispensable for the primary formation of common lymphoid progenitors, while other factors, such as E2A, early B cell factor 1 (Ebf1), Pax5, and forkhead box protein 1 (Foxo1), have important roles in the B cell–specific gene expression program (Nutt and Kee, 2007; Lin et al., 2010). Foxo1 transcriptionally up-regulates *IL7R* expression, controlling proliferation and apoptosis of pro–B cells after IL-7 stimulation (Milne and Paige, 2006; Dengler et al., 2008; Ochiai et al., 2012). During recombination of the *IgH* locus, Foxo1 and Foxo3A activate recombination-activating gene proteins 1 and 2 (Rag1 and Rag2), initiating *DH*-to-*JH* rearrangements on both *IgH* alleles, followed by *VH*-to-*DJH* rearrangements (Herzog et al., 2009; Clark et al., 2014). After successful *VDJ* recombination in IL-7–responsive pro–B cells, a μ heavy chain together with the surrogate light chain forms the pre–B cell receptor (pre-BCR) and pro–B cells develop into large pre–B cells, which become desensitized to IL-7 (Marshall et al., 1998). After a clonal expansion phase (Melchers, 1995; Herzog et al., 2009), large pre–B cells develop into small pre–B cells where *VJ* rearrangement on the light chain locus starts and cells stop to proliferate. The transition from large to small pre–B cells is regulated by interferon regulatory factors 4 and 8 (Irf4 and Irf8), which induce *Ikaros* and *Aiolos* expression (Ma et al., 2008). Both Irfs promote light chain rearrangement and transcription, either through direct activation of Ig light chain enhancers or indirectly through attenuation of IL-7 signaling. During the attenuation of IL-7 signaling, the transcription factor Ikaros is mandatory for the differentiation of large pre–B cells to small B cells, limiting large pre–B cell expansion by directly inhibiting the G1-S transition (Joshi et al., 2014; Schwickert et al., 2014).

Apart from the Foxo1 and Irfs transcription factors, the activator protein 1 (AP-1) family belonging to the dimeric basic region-leucine zipper transcription factors has been proposed to be important for B cell function (Karin et al., 1997). Hetero- or homodimers of Jun (c-Jun, JunB, JunD) and Fos (cFos, FosB, Fra-1, Fra-2) complexes can regulate the expression of a multitude of genes, leading to regulation of cell proliferation, apoptosis, and differentiation (Liebermann et al., 1998). In B cells, increased expression of JunB, JunD, FosB, and Fra-1 was detected after the stimulation of primary B cells through the surface BCR and/or the CD40 receptor (Tilzey et al., 1991; Huo and Rothstein, 1995, 1996). Recently, Fra-1 was found to limit plasma cell differentiation and exacerbation of antibody responses in mice (Grötsch et al., 2014). In several models, Fra-2 was shown to regulate differentiation and proliferation of cells (Lawson et al., 2009; Bozec et al., 2010). Despite the similar structure between Fra-1 and Fra-2, these two proteins have distinct target genes (Eferl et al., 2004; Bozec et al., 2010). In B cells, the role of Fra-2 re-

*D. Mielenz and A. Bozec contributed equally to this paper.

Correspondence to Aline Bozec: aline.bozec@uk-erlangen.de

Abbreviations used: BCR, B cell receptor; ChIP, chromatin immunoprecipitation; ChIP-seq, ChIP sequencing; Ebf, early B cell factor; Foxo, forkhead box protein; Irf, interferon regulatory factor; VCAM, vascular cell adhesion protein.

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mains to be determined. We hypothesized that Fra-2 deletion in B cells could regulate B lymphocyte development and activation independently of Fra-1. To determine the influence of Fra-2 in the B lineage, we crossed Mb1-Cre mice (Hobeika et al., 2006) with Fra-2 floxed mice (Eferl et al., 2007). The deletion of Fra-2 severely reduced the number of B cells in bone marrow and spleen, leading to decreased basal levels of circulating Igs. Interestingly, we demonstrated that Fra-2–deficient bone marrow B cells display strong reductions of *Irf4* and *Foxo1* transcript levels. A genome-wide analysis of Fra-2 occupancy revealed a complex regulatory network whereby Fra-2 induces B cell proliferation and differentiation. Our data identified Fra-2 as a key regulator of *Irf4* and *Foxo1* and their downstream targets *Ikaros*, *IL7R*, and *Rag 1* and *2*.

RESULTS

Expression of Fra-2 on B cell subsets

To determine whether Fra-2 is an important transcription factor during B cell development, we first quantified its transcript levels in B cell subsets sorted from bone marrow and spleen as well as after in vitro B cell stimulation. Fra-2 is expressed in all B cell subsets analyzed (Fig. S1 a). On one hand, its mRNA levels decreased after LPS and anti-CD40/BCR/IL-4 stimulations of marginal, follicular, and B1 B cells (Fig. S1 b). On the other hand, *Fra-2* mRNA was up-regulated in pro–B cells after 3 and 6 h of IL-7 stimulation (Fig. S1 c). Therefore, to investigate Fra-2 function during B cell development, we generated B cell–specifically Fra-2 deleted mice. Mb1-Cre mice (Hobeika et al., 2006) were crossed with mice carrying *Fra-2 floxed* alleles (Eferl et al., 2007) to delete Fra-2 (Fra-$2^{\Delta B\ cell}$) in B lymphocytes (Fig. S1 d). A mean of 70–80% deletion of *Fra-2* on the mRNA level was observed in sorted B cell subsets from bone marrow and spleen from mutant mice compared with littermate control (Fig. S1 a).

Next, we assessed whether Fra-2 regulates B cell numbers in the bone marrow and affects B cell proliferation and transcription factor expression. Indeed, flow cytometric analyses of 6-wk-old Fra-$2^{\Delta B\ cell}$ mice showed a decreased number of pro, pre, immature, and mature B cells in the bone marrow (Fig. 1, a–c). To determine the cause of the decreased B cell numbers in Fra-2–deficient mice, cell cycle analysis was performed in pro– and pre–B cells from bone marrow of Fra-$2^{\Delta B\ cell}$ and littermate control mice injected in vivo with BrdU. Fra-$2^{\Delta B\ cell}$ pro–B cells showed a significant reduction in G0/G1 phase compared with littermate mice (Fig. 1 d). Fra-$2^{\Delta B\ cell}$ pre–B cells accumulated in the G0/G1 phases of the cell cycle, with a significant reduction in the S phase when compared with pre–B cells from littermate mice (Fig. 1 d), suggesting a defect in the proliferation of Fra-2–deficient pre–B cells. Gene profiling analysis in bone marrow B cells from littermate and Fra-$2^{\Delta B\ cell}$ mice revealed that mRNA levels of molecular markers essential for B cell development and proliferation, such as *Ebf1*, *Foxo1*, *Irf4*, *Ikaros*, *Aiolos*, *IL7Ra*, *Rag1*, and *Rag2* mRNAs, were decreased, whereas no changes were detected for *Pu1*, *SpiB*, *Irf8*, *IgII1*, and *VpreB* mRNA levels (Fig. 1 e). Altogether, these data suggest that Fra-2 regulates early B cell proliferation and, thereby, the output of differentiated B cells from the bone marrow, by controlling the expression of essential B cell transcription factors.

Decreased spleen B cell numbers in Fra-2–deficient mice

The decreased B cell numbers in the bone marrow of Fra-2–deficient mice prompted us to characterize peripheral B cell populations in the spleen. As shown in Fig. 2 a, the percentage and number of transitional T1 and T2 B cells remained unchanged in Fra-2–deficient mice compared with littermate controls. However, marginal and follicular B cell numbers were significantly decreased in Fra-$2^{\Delta B\ cell}$ spleens (Fig. 2 b). Staining of IgM and IgD showed reduced numbers of follicular and marginal-zone B cells in spleens from mutant mice compared with littermate control (Fig. 2 c). The follicular architecture of Fra-$2^{\Delta B\ cell}$ spleen appeared essentially normal (Fig. 2 d). Collectively, targeted deletion of Fra-2 in B cells appears to control the number of B2 B cells. In line with reduced B cell numbers, basal IgG1, IgG2b, and IgM titers were reduced (Fig. 2 e).

Impaired in vitro transition of large pre–B cells from Fra-$2^{\Delta B\ cell}$ mice to immature B cells

To determine the mechanism leading to decreased B cell numbers in Fra-2–deficient mice, pro–B cells isolated through cell sorting were stimulated with IL-7 (Milne et al., 2004). Differentiation of pro–B cells/large pre–B cells into small pre–B cells and immature B cells was significantly decreased at high IL-7 (5 ng/ml; Fig. 3, a and b). At low IL-7 concentrations (0.5 ng/ml), differentiation of Fra-2–deficient pro–B cells/large pre–B cells into small pre–B cells was comparable with littermate control cells, and development of Fra-2–deficient immature B cells was partially rescued (Fig. S2). In addition, the proliferation of primary Fra-2–deficient pro–B cells in response to 72-h stimulation with 5 ng/ml IL-7 was decreased (Fig. 3 c) and was pinpointed to a significant decrease in DNA replication compared with littermate control pro/pre–B cell cultures (Fig. 3 d). These data are in accordance with our in vivo data (Fig. 1 d), as we applied conditions under which most pro–B cells express cytoplasmic IgM and have the capability to become pre–B cells (Marshall et al., 1998). There was no effect of *Fra-2* deletion on apoptosis under these conditions (Fig. 3 e). Moreover, mature mutant and littermate control B cells proliferated similarly after stimulation with LPS or CD40L/αBCR/IL-4, suggesting a specific involvement of Fra-2 in IL-7–driven proliferation (Fig. S4, a and b).

Next, molecular analyses were performed by quantitative PCR profiling. As previously found in total bone marrow B cells in vivo (Fig. 1 e), mutant pro–B cells showed decreased expression of *Ebf1*, *Foxo1*, *IL7Rα*, *Irf4*, *Ikaros*, *Aiolos*, and *Rag1/2* mRNA expression compared with littermate control pro–B cells (Fig. 3 f). Reduced *Foxo1* and *Rag1/2* transcripts are fully compatible with both defects in proliferation and differentiation of pre–B cells (Amin and Schlissel,

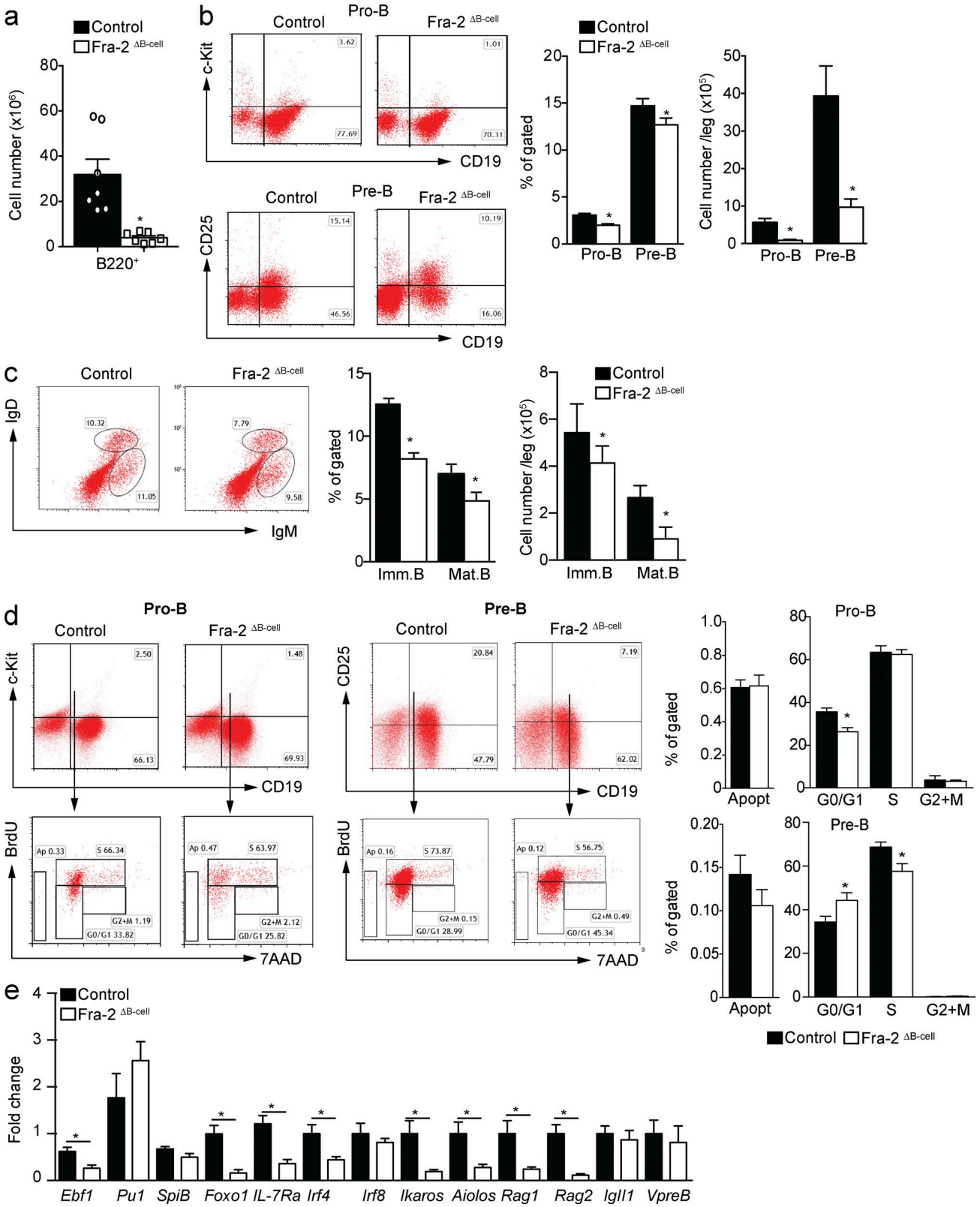


Figure 1. **Fra-2$^{\Delta B\ cell}$ mice have decreased B cell numbers in the bone marrow.** (a–c) Absolute number of total B cells: (a), flow cytometric analysis and absolute number of pro–B cell and pre–B cells (b), and immature (imm.) and mature (mat.) B cells (c) from Fra-2$^{\Delta B\ cell}$ and littermate control mice (n = 5). (d) Flow cytometry analysis of cell cycle phases (G0/G1, S, and G2+M) and apoptosis (apopt) 24 h after in vivo BrdU incorporation in pro– and pre–B cells

2008; Dengler et al., 2008). Because Ikaros deficiency induces pronounced cellular adherence (Schwickert et al., 2014), we assessed pro–B cell adherence in the presence of CXCL2 and IL-7. Indeed, Fra-2$^{\Delta B\ cell}$ cells showed twofold increased adherence compared with control pro–B cells to vascular cell adhesion protein 1 (VCAM-1)–coated surfaces (Fig. 3 g). Collectively, these results suggest that Fra-2 plays an important role in the proliferation, adhesion, and differentiation of pro– and pre–B cells, likely by regulating the expression of essential transcription factors such as *Ebf1*, *Ikaros*, *Foxo1*, *IL7Rα*, *Irf4*, and *Rag1/2*.

Genomic targeting analysis of Fra-2 in bone marrow isolated B cells

To determine the genomic binding pattern of Fra-2 in an unbiased manner, we isolated B220^{+} B cells from bone marrow of Fra-2$^{\Delta B\ cell}$ and littermate control mice and performed chromatin immunoprecipitation (ChIP) with Fra-2 antibody. ChIP sequencing (ChIP-seq) analyses identified consensus TRE elements of AP-1/Fra-2-binding motifs in B cells (Fig. S3 a). ChIP-seq peaks identified molecular markers related to B cell development, such as *Ebf1* (Fig. 4 a), and cell cycle, BCR response, or cell proliferation, such as *pdk3*, *lyn37*, and *miR125-1b* (Fig. S3 b and not depicted). In accordance with Fra-2 binding to these specific targets genes, the mRNA levels of *miR125-1b*, *lyn37*, and *pdk3* were decreased in Fra-2$^{\Delta B\ cell}$ B cells compared with littermate controls (Fig. 4 b), suggesting that Fra-2 regulates B cell proliferation by a direct transcriptional activation of multiples target genes. Because *Irf4* and *Foxo1* expression was found to be down-regulated in vivo and in vitro in Fra-2–deficient B cells, we first analyzed the potential binding of Fra-2 and other AP-1 members on *Irf4* and *Foxo1* promoters (Fig. 4 c). Indeed, Fra-1, Fra-2, and Jun proteins were able to bind the *Irf4* promoter on a TRE element located at −1,986 bp but not −3,429 bp (Fig. 4 c and not depicted). Moreover, ChIP analyses of the AP-1 members in Fra-2–deficient cells revealed that c-Jun, JunD, and JunB are the potential dimerization partners of Fra-2 on the *Irf4* promoter and that Fra-1 cannot compensate for the loss of Fra-2 (Fig. 4 c). Furthermore, we could also show that Fra-2 binds the *Ikaros* promoter on the known Irf4 sites −235 bp (Fig. 4 d). In accordance, Fra-2 and Irf4 binding on the *Ikaros* promoter was reduced in Fra-2–deficient cells compared with littermate control (Fig. 4 d), suggesting a possible physical interaction between Fra-2 and Irf4. The consensus TRE elements of Fra-2–binding motifs in B cells revealed that Irf4 might be one of its cofactors (Fig. S3 a). Next, we tested the Irf4-Fra-2 interaction by coimmunoprecipitation in littermate control and Fra-2–deficient B cells. In fact, Irf4 interacts with Fra-2, as shown by coimmunoprecipitation (Fig. 4 e). In addition, Western blotting analysis showed a reduction of Irf4 levels in Fra-2–deficient cells (Fig. 4 f). All together, these data suggest that an interaction of Fra-2 with Irf4 could be essential for Irf4 action on the *Ikaros* promoter.

Next, we determined whether similar results could be obtained with the *Foxo1* promoter. ChIP analyses showed that AP-1 proteins bound to the *Foxo1* promoter at the −1,270-bp site and that cJun, JunB, and JunD were the potential dimerization partners of Fra-2 on the *Foxo1* promoter (Fig. 4, g and h). Moreover, in the absence of Fra-2, Foxo1 showed a reduction of its binding on the promoter of its target genes IL-7 receptor (Fig. 4 i) and Rag (Fig. 4 j). In addition, Foxo1 protein was also decreased in B cells deficient for Fra-2 (Fig. 4 f). Altogether, these results indicate that Fra-2 transcriptionally regulates *Irf4* and *Foxo1*, which through the regulation of their downstream targets *Ikaros*, *IL7Ra*, and *Rag 1/2* control B cell development (Fig. 4 k).

Foxo1 and Irf4 overexpression rescued the proliferation defect of Fra-2$^{\Delta B\ cell}$ B cell

To determine whether the reduced levels of Foxo1 or Irf4 proteins were responsible for the decreased B cell numbers in Fra-2–deficient mice and the proliferation defect in response to IL-7, restoration of *Irf4* and *Foxo1* expression was performed in vitro. Indeed, reexpression of *Foxo1* or *Irf4* by retroviral transduction in IL-7–stimulated Fra-2$^{\Delta B\ cell}$ pro–B cells (Fig. S4 a) led to full rescue of pre–B cell differentiation (Fig. 5 and Fig. S4 b). As expected, the number of small pre–B cells and immature B cells was lower in Fra-2$^{\Delta B\ cell}$ B cells compared with controls after infection with the control vector (Fig. 5, a and b). However, the frequency of Fra-2$^{\Delta B\ cell}$ small pre–B cells was rescued by retroviral transduction of Foxo1 and Irf4. Although the number of immature B cells in Fra-2$^{\Delta B\ cell}$ B cells was still decreased after reexpression of *Foxo1* (Fig. 5, a and b), Fra-2$^{\Delta B\ cell}$ pro–B cells expressing *Foxo1* or *Irf4* showed rescued proliferation (Fig. 5 c). These data indicate that Fra-2–mediated expression of *Foxo1* is required, probably via Irf4, but not sufficient for immature B cell differentiation. Moreover, mRNA expression levels of *Ikaros*, *Aiolos*, and *Rag2* were fully recovered after *Irf4* and *Foxo1* overexpression, while the expression of *Rag1* was still decreased in Fra-2$^{\Delta B\ cell}$ pro–B cells after *Foxo1* expression (Fig. 5 d and Fig. S5). The incomplete recovery of *Rag1* expression could account for the incomplete rescue of immature B cells. Altogether, these data suggest that Irf4 and Foxo1 are responsible for B cell proliferation and differentiation arrest in Fra-2–deficient cells.

from Fra-2$^{\Delta B\ cell}$ and littermate control mice (n = 5). (e) Real-time PCR analyses of *Ebf1*, *Pu1*, *SpiB*, *Foxo1*, *IL7R*, *Irf4*, *Irf8*, *Ikaros*, *Aiolos*, *Rag1*, *Rag2*, *Igll1*, *and VpreB* in B220^{+} bone marrow B cells from Fra-2$^{\Delta B\ cell}$ and littermate control mice (n = 5). Bars represent mean values ± SD. Data show one representative out of three independent experiments. Statistical analysis was performed using Student's t test; *, $P < 0.05$.

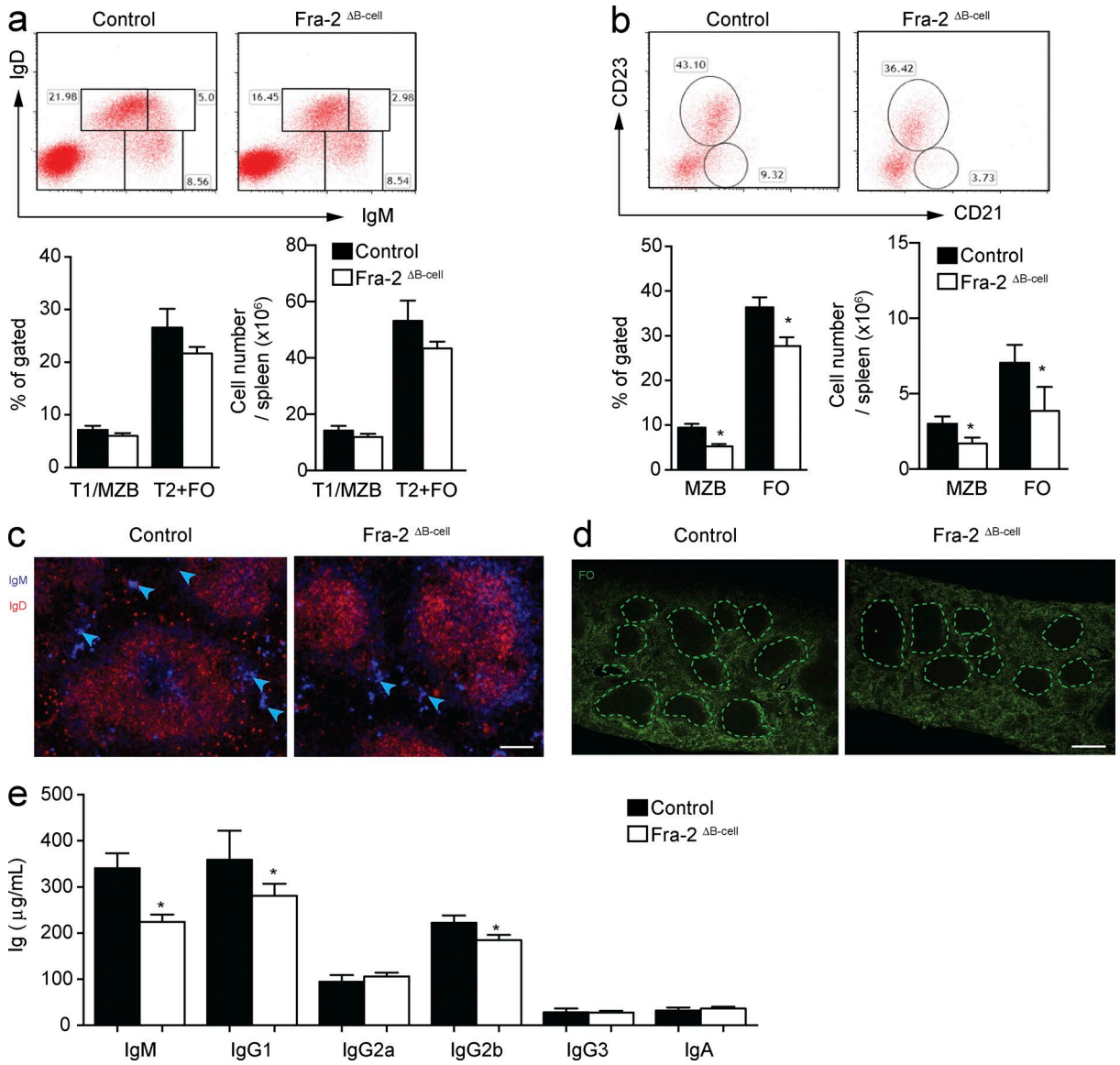


Figure 2. **B cell populations in the spleen are also decreased in Fra-2$^{\Delta B\ cell}$ mice.** (a and b) Flow cytometry analyses of T1/MZB, T2+FO B cells (a), MZB and FO B cells (b) in spleen from Fra-2$^{\Delta B\ cell}$ and littermate control mice ($n = 5$). (c and d) Stainings of IgD/IgM (c; bar, 200 µm) and FO B cells (d; bar, 800 µm) in spleen of Fra-2$^{\Delta B\ cell}$ and littermate control mice. The blue arrowheads indicate IgM$^+$ cells. FO, follicular B cell based on CD23 staining. (e) Basal levels of Ig subclasses (IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA) in Fra-2$^{\Delta B\ cell}$ and littermate control sera ($n = 5$). Bars represent mean values ± SD ($n = 5$). Statistical analysis was performed using Student's *t* test; *, $P < 0.05$.

DISCUSSION

In this study, we reveal a hitherto unknown role for the Fra-2/AP-1 transcription factor in early and peripheral B cell development at several hierarchical levels. First, Fra-2 controls proliferation of B220$^+$ B cells in the bone marrow and proliferation of pro–B cells in IL-7–stimulated cultures. Second, Fra-2 controls the differentiation step from large to small pre–B cells. Third, Fra-2 controls the differentiation of immature B cells, and fourth, Fra-2 controls the generation of B2 B cells from T2 precursors in the spleen. Fra-2 exerts its effects through two mechanisms: by binding and thereby controlling the expression of several transcription factors, such as *Ebf1*, *Foxo1*, *Irf4*, *and Ikaros*, endowed in B cell development and differentiation and by interacting with Irf4 to regulate Irf4 target genes. In accordance with Fra-2–mediated control of expression of *Foxo1* and *Irf4*, the transcription of their target genes *Aiolos*, *Ikaros*, *Rag 1/2*, and *IL7Rα* is also down-regulated in the absence of Fra-2, thereby explaining the role of Fra-2 in pro–B cell proliferation and pre–B cell differentiation (Fig. 5 e).

B cell development is tightly controlled by the plural action of transcription factors able to guide B cell commit-

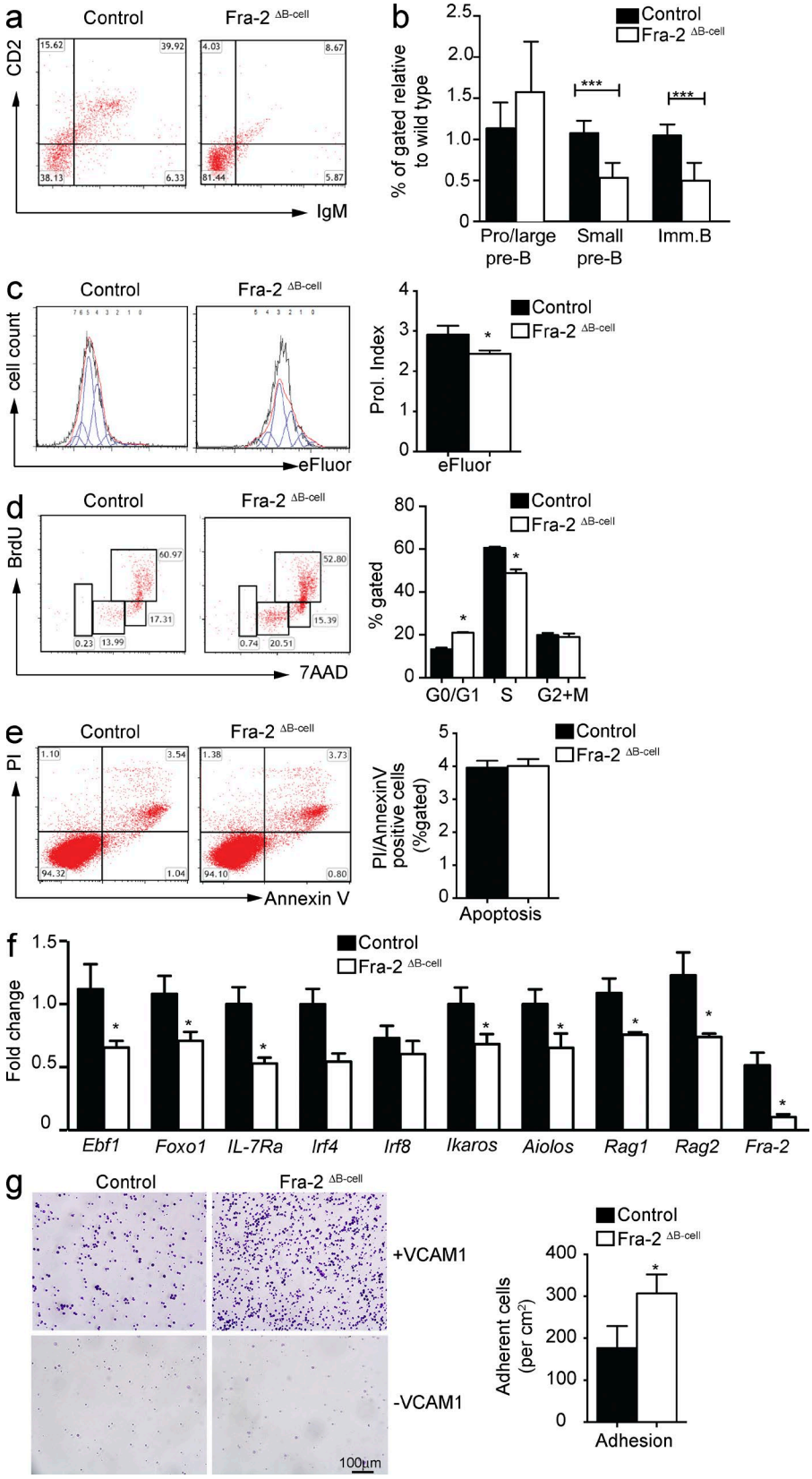
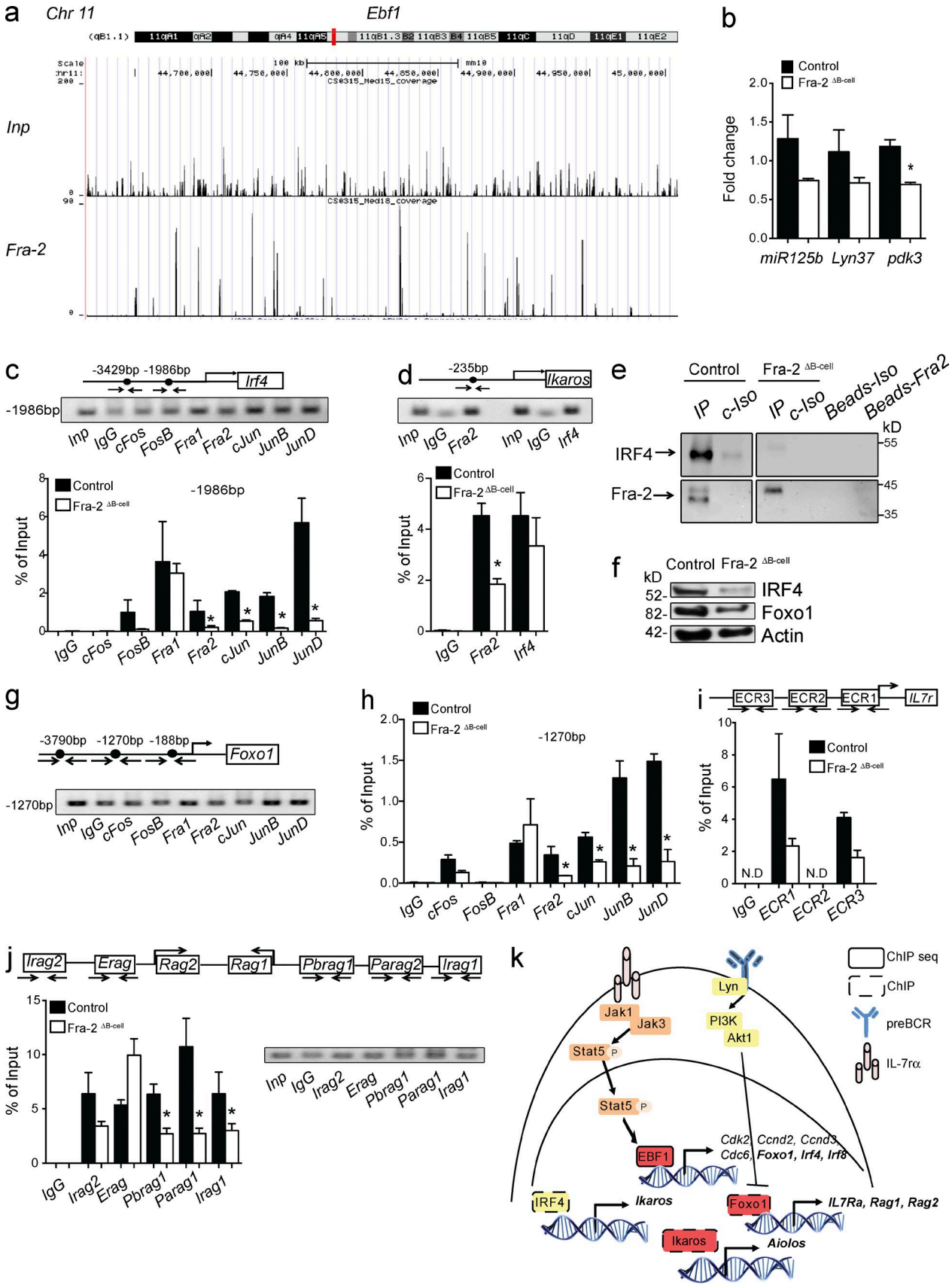


Figure 3. **Fra-2 as a positive regulator of B cells proliferation in vitro.** (a and b) Flow cytometry analysis of pro–/large pre–B cells, small pre–B cells, and immature B cells after 5 d of stimulation with IL-7 (pooled data from three independent experiments, littermate control cells set as reference). (c) Flow cytometry analysis of the proliferation (prol.) rate of sorted pro–B cells stimulated for 5 d with IL-7 (5 ng/ml). Red is the draw model, blue the draw model component, and gray the total division profile. (d and e) Flow cytometry analysis of cell cycle G0/G1, S, and G2+M phases by thymidine analogue BrdU incorporation and DNA content (d) and percentage of apoptotic cells (e) in pro–B cell cultures of Fra-$2^{\Delta B\ cell}$ and littermate control cells stimulated with IL-7. (f) Real-time PCR analyses of *Foxo1*, *Irf4*, *Ikaros*, *Aiolos*, *Rag1*, *and Rag2* in pro–B cell culture of Fra-$2^{\Delta B\ cell}$ and littermate control cells stimulated with IL-7. (g) adhesion analyses of pro–B cells plated in coated with VCAM-1 wells and stimulated for 6 h with CXCL12 and IL-7. Bars represent mean values ± SD. Data show one representative out of three independent experiments. Statistical analysis was performed using Student's *t* test; *, $P < 0.05$; ***, $P < 0.001$.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Cdc6, Foxo1, Irf4, Irf8
IRF4
Ikaros
Foxo1
IL7Ra, Rag1, Rag2
Ikaros
Aiolos

ment, survival, proliferation, Ig rearrangement, receptor editing, and final differentiation into plasma cells (Nutt and Kee, 2007; Dengler et al., 2008; Ochiai et al., 2012). Fra-2 down-regulation in B cells leads to reduced numbers of pro, pre, immature, and mature B cells in the bone marrow because of impaired B cell proliferation as measured by BrdU incorporation of pro– and pre–B cells. Within the B cell lineage only pro– and pre–B cells divide in the bone marrow (Carsetti, 2000). The reduced number of B cells in Fra-2–deficient bone marrow was likely due to decreased numbers of pro–B cells in the G1/G0 phase and decreased numbers of pre–B cells in the S phase of the cell cycle. These data strongly imply a function of Fra-2 in B cell proliferation, similar to other members of the AP-1 family previously shown to positively regulate proliferation in other cell types (Shaulian and Karin, 2001; Mercer et al., 2011). The effects exerted by Fra-2 were also directly measured in IL-7–stimulated B cell cultures in vitro. It is possible that deletion of *Fra-2* rendered pro/pre–B cells refractory to IL-7, as we showed that Fra-2 also up-regulates Foxo1, which in turn promotes *IL7Rα* expression (Ochiai et al., 2012). Importantly, we demonstrated that reconstitution of *Foxo1* expression in Fra-2–deficient pro/pre–B cells restored responsiveness to IL-7 again. In addition to a direct effect of Fra-2 on IL-7 induced proliferation, we revealed that Fra-2 decreased adhesion of pro–B cells to VCAM-1. In vivo, Fra-2 might therefore be required to support mobilization of pro–B cells toward IL-7 "high" niches during their development (Park et al., 2013). Interestingly, Fra-2 was up-regulated in pro–B cells after IL-7 stimulation by an as yet unidentified pathway. As Fra-2 controls *IL7Rα* expression and vice versa, up-regulation of Fra-2 by IL-7 constitutes a positive feedback loop supporting the proliferation of pro–B cells because IL-7 is a cytokine essential for lymphocyte development and proliferation (Kittipatarin and Khaled, 2007). In T cells, IL-7 promotes JNK activation and induces the expression of *JunD*, another member of AP-1 transcription factor (Ruppert et al., 2012), which supports our notion of JunD as partner of Fra-2 on the Irf4 promoter.

A reduction of *Fra-2* expression in mature B cells was observed after LPS or CD40L/BCR/IL-4 stimulation, suggesting a specific role of Fra-2 during IL-7 signaling. Indeed, each AP-1 transcription factor might have time- and signal-dependent action in B cells (Ohkubo et al., 2005; Grötsch et al., 2014). As a consequence of the Fra-2/IL-7 loop, we hypothesize that each AP-1 transcription factor has a specific action on B cell physiology by the regulation of a network of target genes. In fact, despite protein similarity between Fra-1 and Fra-2, their action seems to be different in B cells, as increased proliferation was reported in mature B cells lacking Fra-1 (Grötsch et al., 2014). Fra-1 was shown to directly regulate *Blimp1* expression at the promoter level in competition with Fos in activated mature B cells. In contrast, loss of Fra-2 affects the expression of genes essential for early B cell development, such as *Ebf1*, *Foxo1*, *Irf4*, *Ikaros*, *Aiolos*, and *Rag1/2*. These data are in accordance with a previous study in which transient expression of *cJun* and *cFos* AP-1 transcription factors preceded increased *Ebf1* and *Foxo1* expression, already suggesting an important role of the AP-1 members in their regulation (Mercer et al., 2011). It was previously shown that Foxo1 is important for both early pro–B cell development and pre–B cell development (Dengler et al., 2008). Moreover, Ikaros is required for the transition of large to small pre–B cells (Lu, 2008; Heizmann et al., 2013; Joshi et al., 2014; Schwickert et al., 2014). Thus, the phenotypes of Fra-$2^{\Delta B\ cell}$ mice resemble those deficient of Foxo1 and Ikaros in the B cell lineage and support our finding that Fra-2 controls *Foxo-1* and *Ikaros* expression. At the molecular level, lack of Foxo1 impaired the expression of *IL7Rα* and *Rag1/2* (Herzog et al., 2009). Intriguingly, we observed decreased *Foxo1*, *IL7Rα*, and *Rag1/2* expression in Fra-$2^{\Delta B\ cell}$ mice, supporting our notion that regulation of *IL7Rα* and *Rag1/2* expression by Fra-2 is mediated via Foxo-1. Fra-2 seems to be upstream in this hierarchy.

In addition, Fra-2 appears to induce a second transcriptional pathway, as expression of *Foxo1* could not fully restore *Rag1* expression and immature B cell differentiation. This pathway could be linked to the up-regulation of Irf4. In support of this notion, we have shown that Fra-2 regulates not only Foxo1 but also Irf4, which exhibit important roles during B cell development (Lu, 2003, Lu, 2008). Irf4/8 double-knockout mice show arrested B cell development at the cycling pre–B cell stage, with absence of immature IgM^+ B cells and high levels of *pre-BCR* expression (Lu et al., 2003). Molecularly, both Irf4 and 8 induce germline transcription and

Figure 4. **Genomic analyses of Fra-2 targets in B cells.** (a) ChIP-seq analysis of the binding of Fra-2 at *Ebf1* promoter ($n = 3$) performed in $B220^+$ B cells from control mice. (b) Real-time PCR analyses of *mir125b*, *lyn37*, and *pdk3* in pro–B cell cultures of Fra-$2^{\Delta B\ cell}$ and littermate control cells stimulated with IL-7 for 5 d ($n = 4$). (c) PCR loading fragments of ChIP analyses from littermate control cells for *Irf4* promoter after immunoprecipitation of the chromatin with AP-1 protein antibodies. Arrows indicate primers amplifying fragments for the TRE elements. Real-time PCR analyses of ChIP in Fra-$2^{\Delta B\ cell}$ and littermate control $B220^+$ B cells for *Irf4* promoter with AP-1 antibodies ($n = 3$). (d) PCR loading fragments of ChIP analysis for *Ikaros* promoter after Fra-2 and Irf4 ChIP and real-time PCR quantification of the binding potential of AP-1 members in Fra-$2^{\Delta B\ cell}$ and littermate control cells ($n = 3$). (e) Coimmunoprecipitation of IRF4 and Fra-2 proteins in $B220^+$ bone marrow B cells ($n = 3$). (f) Western blot analysis of Irf4, Foxo1, and actin proteins in Fra-$2^{\Delta B\ cell}$ and littermate control B cells ($n = 3$). (g and h) PCR loading fragments of ChIP analysis of *Foxo1* promoter after AP-1 antibody ChIP (g) and real-time PCR quantification (h). (i and j) Real-time PCR quantification of ChIP analysis for *IL7Rα* (i) and (j) promoters after ChIP with Foxo1 antibody in Fra-$2^{\Delta B\ cell}$ and littermate control B cells. (k) Schematic representation of genes transcriptionally regulated by Fra-2. pBCR, pre-BCR. Bars represent mean values ± SD ($n = 3$). Data show one representative out of three independent experiments. Statistical analysis was performed using Student's *t* test; *, $P < 0.05$. Beads-Fra-2, beads treated with Fra-2 antibody; Beads-Iso, beads treated with control isotype antibody; c-Iso, control isotype antibody; Inp, input; IP, immunoprecipitation with Fra-2 antibody.

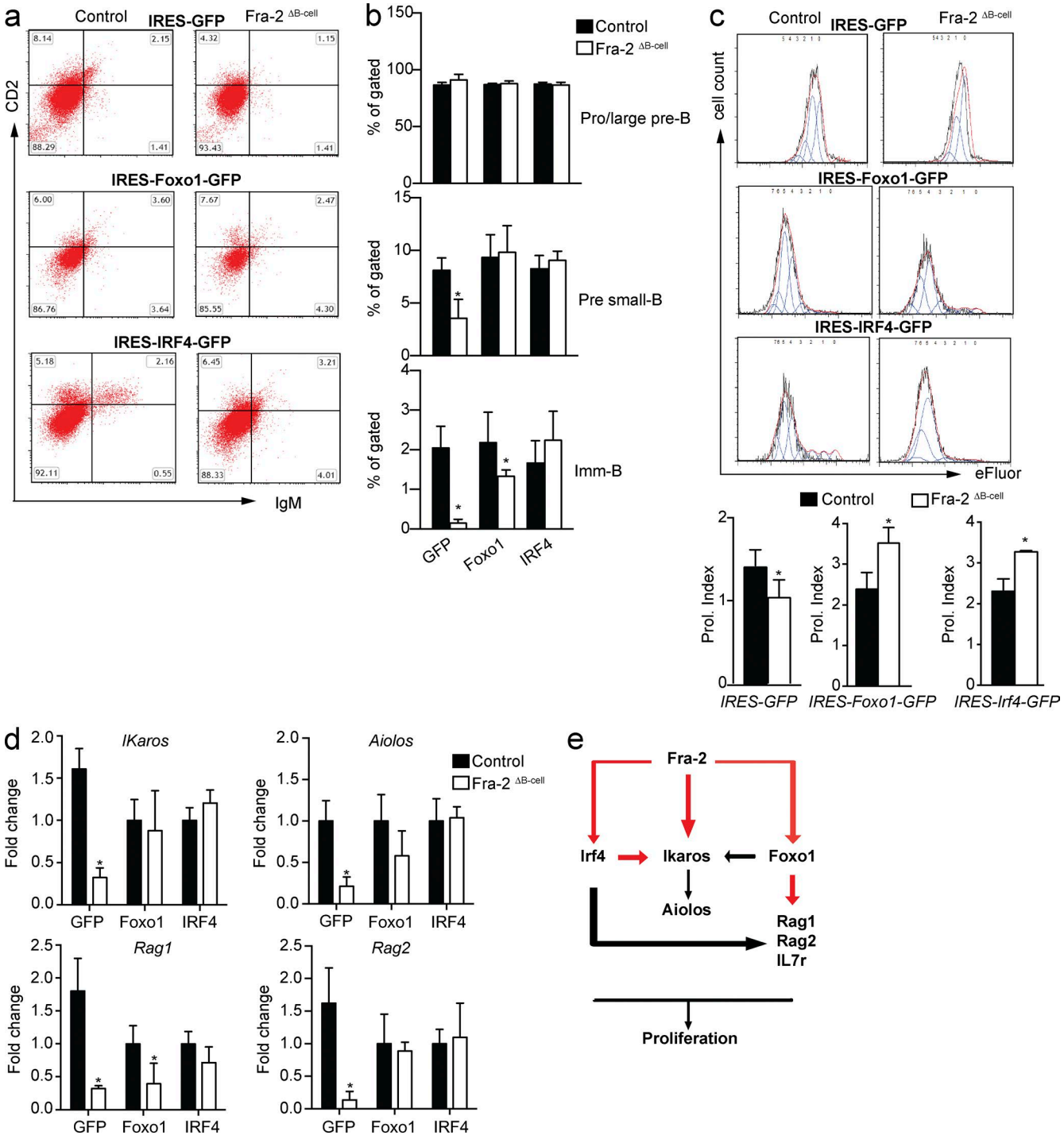

Figure 5. ***Foxo1*and *IRF4* overexpression rescued the proliferation defect of Fra-2$^{\Delta B\ cell}$ B cells in vitro.** (a and b) Flow cytometry analysis of GFP$^+$ gated pro/large pre–B cells, small pre–B cells, and immature B cells after infection with IRES-GFP, IRES-Foxo1-GFP, and IRES-Irf4-GFP expressing retrovirus and stimulation with IL-7 for 5 d ($n = 5$). (c) Flow cytometry analysis of the proliferation rate in Fra-2$^{\Delta B\ cell}$ and littermate control pro–B cells after retrovirus infection ($n = 5$). (d) Real-time PCR analysis of *Ikaros*, *Aiolos*, *Rag1*, *and Rag2* genes of pro–B cell culture after infection with IRES-GFP, IRES-Foxo1-GFP, and IRES-Irf4-GFP expressing retrovirus and after stimulation with IL-7 for 5 d. GFP controls were set to 1 ($n = 5$). (e) Scheme of molecular mechanism of how Fra-2 acts on B cells. Red arrows indicate new findings, and black arrows indicate previous publication mechanisms. Bars represent mean values ± SD. Data show one representative out of three independent experiments. Statistical analysis was performed using Student's *t* test; *, $P < 0.05$.

promote *L chain* rearrangement and transcription (Ma et al., 2008). Interestingly, when looking at downstream targets of Foxo1 and Irf4 by ChIP analyses, we showed that (1) Fra-2 together with Jun proteins bound at *Foxo1* and *Irf4* promoters, (2) Fra-2 together with Irf4 bound to the *Ikaros* promoter, and (3) Irf4 induced *Ikaros* and *Aiolos* expression in pre–B cells dependent of Fra-2. These findings add another level of regulation to the growth inhibition and cell cycle arrest induced by Irf4 or the regulation of pre-BCR and the cell cycle withdrawal mediated by *Ikaros*, *Aiolos*, and *Irf4*, respectively (Ma et al., 2008). Functionally, we could also link the increased adherence of Fra-$2^{\Delta B\ cell}$ B cell to the inactivation of *Ikaros*, which exhibits aberrant pro–B cells characterized by increased cell adhesion (Schwickert et al., 2014). The impaired down-regulation of the surrogate chain and the decreased expression of *κ light chain* found in Fra-$2^{\Delta B\ cell}$ mice (unpublished data) could therefore be directly dependent on the positive regulation of Irf4 transcription by Fra-2; however, additional studies are necessary to further characterize this phenotype. In accordance with impaired *Ig* rearrangement due to decreased *Rag1/2* expression, reduced pro–B cell proliferation, and impaired small pre–B cell differentiation, Fra-$2^{\Delta B\ cell}$ mice presented reduced peripheral B cell numbers and concomitantly reduced abundance of serum Igs. The genomic network governed by Fra-2 extends beyond the rather well-characterized factors Foxo1 and Irf4, as shown by our genomic ChIP-seq analysis of the transcriptional Fra-2 network in B cells. The positive regulation of Irf4 by Fra-2 can also explain the differentiation defect of marginal-zone B cells in Fra-$2^{\Delta B\ cell}$ mice, as impaired marginal-zone B cell differentiation is also seen in B cells from Irf4 knockout mice.

In summary, we demonstrate that Fra-2 acts as a key enhancer of B cell proliferation and differentiation. Fra-2 regulates B cell proliferation by specifically binding to *Foxo1* and *Irf4* promoters, inducing their expression. Fra-2 regulation of Foxo1 and Irf4 is essential for the downstream activation of *Ikaros*, *Aiolos*, and *Rag1/2* expression, thereby regulating early B cell proliferation and differentiation.

MATERIALS AND METHODS

Animals

To generate B cell–specific Fra-2 deleted mice, *Fra-2 floxed* mice (Eferl et al., 2007) were crossed with Mb1-*cre* mice (129S-C57BL/6 mixed background; Hobeika et al., 2006). All mice were maintained on a mixed C57BL/6 background and in a specific pathogen–free facility. Littermate mice (Fra-2 *+/+* Mb1-*cre tg* and Fra-2 *floxed Mb1-Cre-*) were used as controls in this study. 6-wk-old male and female mice were used in all experiments. Genotyping primers were as follows: Fra-2 lox forward: 5′-GAGGGAGTTGGGGATAGAGTG GTA-3′; Fra-2 lox reverse: 5′-GGACAGCAGGTCAGG AGTAGATGA-3′; Mb1Cre forward: 5′-ACCTCTGAT GAAGTCAGGAAGAAC-3′; and Cre reverse: 5′-GGAGAT GTCCTTCACTCTGATTCT-3′. Animal experiments were approved by the local ethics committee of Regierung von Mittelfranken, Germany.

Antibodies, flow cytometry, and cell sorting

The following antibodies were used for flow cytometry: Alexa Fluor 647–conjugated anti-CD19, FITC-conjugated anti-CD21/CD35, phycoerythrin (PE)–conjugated anti-CD23, FITC-conjugated anti-CD45R (B220), allophycocyanin-conjugated anti-CD138, PE-conjugated streptavidin (all from BD), PE-conjugated anti-kappa (Antikörper-online.de), PE-conjugated anti-lambda (Antikörper-online.de), PE-conjugated anti-CD25 (BioLegend), PE-conjugated anti-CD2 (BioLegend), PerCP-conjugated anti-CD45R (B220; Miltenyi Biotec), bioconjugated anti-IgM (eBioscience), anti-CD117 (c-Kit; eBioscience), FITC-conjugated anti-CD2 (eBioscience), FITC-conjugated anti-IgD (SouthernBiotech), and Cy5-conjugated anti-IgM (SouthernBiotech). Analyses of expression of cell surface molecules on a single cell level were performed by flow cytometry with a FACSCalibur (BD) or Gallios (Beckman Coulter) flow cytometer. Cells from bone marrow (1.2×10^6) or spleen (2×10^6) were stained with sufficient amounts of FACS antibodies for 20 min at 4°C in the dark. Afterward, cells were washed with 1 ml FACS buffer (2% FCS and PBS) and suspended in 300 µl FACS buffer or stained with a secondary antibody. For analysis of intracellular proteins, cells were fixed and permeabilized with the Fix&Perm kit (An der Grub) according to the manufacturer's instructions, stained, and analyzed as mentioned before.

For cell sorting, bone marrow cells or splenocytes were incubated in 1 ml antibody solution in 2% FCS and 2 mM EDTA in PBS buffer (MACS buffer) for 30 min at 4°C and then sorted as follows: pro–B cells $B220^+/c\text{-}Kit^+/CD19^+$, T1 B cells $CD23^-/IgM^+/CD21^-$, T2 B cells $CD23^+/CD21^+/IgM^+$, follicular B cells, $IgM^{low}/CD23^+/CD21^+$; MZ B cells, $CD23^-/IgM^+/CD21^+$. Cells were sorted using a MoFlo cell sorter (Dako). Purities of 98–99% were generally achieved.

Isolation of B cells from bone marrow and spleen

Bone marrow was flushed with PBS. Spleens were digested in 5 ml Hank's buffer supplemented with 1 mg/ml collagenase D (Roche) and 200 U/ml DNase I (Roche) for 30 min at 37°C. The reaction was stopped with 150 µl 0.5 M EDTA, and the tissue was squashed through a 70-µm cell strainer before single-cell suspension. Cells from both organs were treated during 5 min with erythrocyte lysis buffer and afterward suspended in 1 ml of in RPMI1640 medium (10% FCS, 1 mM pyruvate, 2 mM l-glutamine, 100 U/ml penicillin/streptomycin, and 50 µM β-mercaptoethanol; Gibco).

In vitro stimulation of pro– and splenic B cells

Pro–B cells were sorted on the basis of their surface markers using a MoFlo cell sorter (Dako), and B cells from spleen were purified by positive selection on CD45R/B220 MACS beads (Miltenyi Biotec). Pro–B cells were cultured in Opti-MEM (10% FCS, 1 mM pyruvate, 2 mM l-glutamine, 100 U/ml penicillin/streptomycin, and 50 µM β-mercaptoethanol; Gibco) and stimulated with IL-7 (5 ng/ml). Splenocytes were resuspended in 90 µl MACS buffer (2% FCS, 0.25 mM

EDTA, and PBS) and 10 µl CD45R/B220 MACS beads (Miltenyi Biotec) per 10^7 cells. After 20 min of incubation at 4°C, cells were washed with MACS buffer and sorted with LS columns. $B220^+$ B cells from spleen were cultured in RPMI1640 (10% FCS, 1 mM pyruvate, 2 mM l-glutamine, 100 U/ml penicillin/streptomycin, and 50 µM β-mercaptoethanol) and stimulated with 10 µg/ml LPS, 10 µg/ml LPS+100 U/ml recombinant mouse IL-4 (Miltenyi Biotec), 10 µg/ml LPS + anti-BCR antibody b.7.6, anti-CD40 mAb (clone FGK), or a mixture of 10 µg/ml anti-BCR antibody b.7.6 anti-CD40 mAb (clone FGK) and IL-4.

Cell cycle analysis

Mice were injected i.p. with 1 mg BrdU for 40 min. Single-cell suspensions were prepared from bone marrow and were stained for cell surface proteins with anti-B220, anti-cKit, anti-CD25, and anti-CD19. Cultured pro–B cells were treated for 45 min with 10 µM BrdU. BrdU was stained with an APC BrdU Flow kit (557892) according to the manufacturer's protocol (BD). Surface-stained cells were fixed, permeabilized, and treated with DNase I. Incorporated BrdU was stained with anti-BrdU, and total DNA was stained with 7-amino-actinomycin D. Single cells were analyzed using a Gallios flow cytometer, and data were evaluated using Kaluza software.

Proliferation analysis and cell death

For proliferation analysis, 5×10^6 pro–B cells or $B220^+$ B cells from spleen were mixed 1:1 with 10 µM eFluor-solution in PBS (cell proliferation dye eFluor 670; eBioscience). Cell solution was incubated for 10 min at 37°C, stopped with 4–5 volumes OptiMEM (10% FCS, 1 mM pyruvate, 2 mM l-glutamine, 100 U/ml penicillin/streptomycin, and 50 µM β-mercaptoethanol) or RPMI1640 medium (10% FCS, 1 mM pyruvate, 2 mM l-glutamine, 100 U/ml penicillin/streptomycin, and 50 µM β-mercaptoethanol) and incubated for 5 min at 4°C. The stained cells were resuspended in OptiMEM or R10+ and either measured at day 0 by flow cytometry or at day 3 after stimulation with IL-7, LPS, or CD40/BCR/IL-4. The number of achieved cell divisions was calculated using the proliferation analysis option in FlowJo or with the software from FACSCalibur. For cell death analysis, stimulated cells were stained with FITC–annexin V for 20 min in annexin V binding buffer (140 mM NaCl, 20 mM Hepes, pH 7.2, and 2.5 mM $CaCl_2$), washed, resuspended in the presence of 200 µl PI, and measured by flow cytometry.

Adhesion assay

12-well plates were coated for 1.5 h at 22°C with VCAM-1 (10 µg/ml; 643-VM, R&D Systems) and washed three times with D-PBS, followed by the addition of 10^5 pro–B cells in 0.5 ml OptiMEM medium containing IL-7 (5 ng/ml) and supplemented with 100 ng/ml CXCL12 (SDF-1α; Sigma-Aldrich). After 6 h of incubation at 37°C, the plates were washed with medium and with D-PBS, followed by fixation for 15 min at 22°C with 4% paraformaldehyde. The plates were washed three times with DPBS and stained for 30 min with 0.1% Crystal Violet and then washed with distilled water and dried. Cells attached to the wells were manually counted as viewed with a microscope. For each genotype, the cells from 12 wells (1.12 cm^2) were counted to determine the cells per square centimeter.

RNA isolation and reverse transcription

Total RNA was isolated with the TRIzol or TriFast protocol (Invitrogen and Peqlab Biotechnologie, respectively). cDNA synthesis was performed using 0.50–2 µg RNA with the cDNA Reagents from Applied Biosystems (Thermo Fisher Scientific).

Real-time PCR analyses

Quantitative PCR reactions were performed using SYBR Green (Eurogentec Deutschland). Primers for genes analyses and primers for ChIP analyses used for real-time PCR are available upon request. The comparative CT method was used to quantify the amplified fragments. RNA expression levels were analyzed in duplicate and normalized to a housekeeping gene such as β-actin, whereas chromatin-bound fragments were normalized to the same fragments amplified from input chromatin.

ChIP

ChIP experiments were performed with ChIP-IT Express kit (Active Motif) according to the manufacturer's protocol. 14 µl FosB, c-Fos, Fra-1, Fra-2, JunB, JunD antibodies (Santa Cruz Biotechnology, Inc.), Irf4 (MUM1; Abcam), cJun, and Foxo1 (Cell Signaling Technology) as well as a rabbit control IgG were used for the immunoprecipitation.

ChIP-seq

DNA was sequenced on an Illumina HiSeq 2500 system, using a single end protocol and 100-bp read length. Reads were aligned to the mm10 mouse reference sequence using bwa version 0.7.8-r455 (Li and Durbin, 2009). Peak calling was performed with MACS version 1.3.7.1 (Zhang et al., 2008) for each of the three Fra2 samples against all three controls. Overlapping peaks from all three Fra2 samples were then used for further analysis. Motif discovery was performed with HOMER version 4.7.2 (Heinz et al., 2010) and a size parameter of 200 bp.

Transient transfection of adherent cells using calcium phosphate

Phoenix-eco cells (4×10^6) were seeded in a 10-cm well plate, 1 d before transfection, and incubated overnight at 37°C and 5% CO_2 in D10 medium. 20 µg DNA was mixed with 125 µl (2 M $CaCl_2$) and 50 µl (10 mM) chloroquine to a final volume of 1 ml with distilled water. Under bubbling conditions, the mixture was added to 1 ml 2× HBS (50 mM Hepes, 10 mM potassium chloride, 12 mM dextrose, 280 mM

sodium chloride, and 1.5 mM Na_2HPO_4, pH 7.4). Precipitates were trickled onto cells, and after 6–8 h of incubation at 37°C and 5% CO_2, cells were provided with 6 ml of fresh D10 medium. Viral supernatant was removed after 24, 48, and 72 h, filtered, and stored at −80°C for infection.

Infection of primary stimulated B lymphoid cells

Pro–B cells (0.5×10^6) were stimulated for 24 h with IL-7 (5 ng/ml) and then infected with retroviral supernatants supplemented with 2 µl transfection reagent (QIAGEN). Infection mixture was centrifuged for 3.5 h at 33,000 rpm at 33°C. Infected cells were stimulated for 3 d with IL-7 and analyzed by flow cytometry.

Coimmunoprecipitation assay

Dynabeads Protein G (Thermo Fisher Scientific) was incubated with 50 µg Fra-2 (Santa Cruz Biotechnology, Inc.) for 10 min at 22°C. Afterward, immunoprecipitation assay was performed with proteins from bone marrow $B220^+$ B cells according to the manufacturer's protocol. In brief, 100 µl of protein solution was incubated for 30 min with the complex Dynabeads Protein G-Fra-2 antibody. The target antigen was eluted under nondenaturing conditions, added 20 ml Elution Buffer (Thermo Fisher Scientific), and incubated with rotation for 2 min at 22°C to dissociate the complex. The eluted protein was stored at −80°C until Western blot analysis.

Western blot

Proteins from bone marrow $B220^+$ B cells or from immunoprecipitation assay were electrophoresed on 10% SDS polyacrylamide gels and transferred to a nitrocellulose membrane (1.5 h, 110 mA). The efficiency of the transfer was determined with Ponceau S solution. Membranes were blocked in 5% milk powder in TBS-T and probed with anti-Irf4 (MUM1; Abcam), anti-Foxo1 (Cell Signaling Technology), and anti–β actin (Sigma-Aldrich) antibodies at 4°C overnight. Membranes were washed in TBS-T (3 × 15 min) and incubated with the secondary antibody anti–mouse/rabbit IgG HRP conjugate (1:20,000; Promega) for 1 h at room temperature. Bands were detected by ECL (Thermo Fisher Scientific).

Statistical analysis

All experiments were repeated at least three times and done in triplicate. All statistical analyses were performed using Prism (GraphPad Software). Statistical analysis was performed using Student's *t* test, and $P < 0.05$ was accepted as significant. Data are shown as means, and error bars represent standard deviations.

Online supplemental material

Fig. S1 depicts expression of *Fra-2* in B cell lineages. Fig. S2 depicts in vitro IL-7 stimulations of B cells from Fra-$2^{\Delta B\ cell}$ and littermate control mice. Fig. S3 shows the Fra-2 recognition sequence in $B220^+$ bone marrow B cells. Fig. S4 shows in vitro LPS or CD40/BCR/IL-4 stimulations of Fra-$2^{\Delta B\ cell}$ and littermate control B cells. Fig. S5 depicts rescue expression of B cell genes.

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