Maintenance of the marginal-zone B cell compartment specifically requires the RNA-binding protein ZFP36L1

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RNA-binding proteins of the ZFP36 family are best known for inhibiting the expression of cytokines through binding to AU-rich elements in the 3′ untranslated region and promoting mRNA decay. Here we identified an indispensable role for ZFP36L1 as the regulator of a post-transcriptional hub that determined the identity of marginal-zone B cells by promoting their proper localization and survival. ZFP36L1 controlled a gene-expression program related to signaling, cell adhesion and locomotion; it achieved this in part by limiting expression of the transcription factors KLF2 and IRF8, which are known to enforce the follicular B cell phenotype. These mechanisms emphasize the importance of integrating transcriptional and post-transcriptional processes by RNA-binding proteins for maintaining cellular identity among closely related cell types.

Marginal-zone (MZ) B cells surround the follicles of the spleen and are continuously exposed to blood-borne antigens. This positioning of MZ B cells enables them to provide immunosurveillance and shuttle antigens to follicular dendritic cells1–2. Their localization to the MZ and their trafficking to and from the B cell follicle also contributes to their survival2–5. Upon encountering antigens, MZ B cells are poised to promote the activation of T cells by presenting antigen as well as differentiating into plasmablasts6. MZ B cells have higher expression of surface immunoglobulin M (IgM), the complement receptors CD35 and CD21 and the lipid-antigen-presenting molecule CD1d than do follicular (Fo) B cells. Together with their elevated expression of Toll-like receptors, these features facilitate rapid responses to blood-borne pathogens such as encapsulated bacteria7,8.

MZ B cells develop through an intermediary population of MZ precursor (MZP) cells that express C-type lectin CD93 (ref. 9). The selection of MZ B cells depends on signaling by membrane immunoglobulins and the receptors Notch2 and BAFF-R7,9. MZ and Fo B cells can be distinguished on the basis of their gene-expression profiles10, and the differences in their transcriptomes contribute to the differential development, localization and function of these cells. Transcriptional regulators have been shown to have specific roles in MZ and Fo B cells. Notch2 is necessary for promotion of the MZ B cell fate11,12. The transcription factor IRF4 and its paralog IRF8 limit the size of MZ B cell pool13, and IRF4 regulates the positioning of cells in the MZ. The transcription factor KLF2 enforces the Fo B cell phenotype. In its absence, the MZ B cell pool is expanded14,15. In contrast, KLF3, which may antagonize KLF2, promotes the development of MZ B cells16. The genes encoding these factors are frequently mutated in splenic MZ lymphoma17, which suggests that they might also form part of a network that sustains the survival and localization of MZ B cells in pathological situations. Thus, the interrelationship among transcriptional regulation, signal transduction and cell positioning for the development and survival of MZ B cells needs to be further understood17.

The post-transcriptional control of RNA regulated by RNA-binding proteins (RBPs) and non-coding RNA complements transcriptional control by adding robustness to gene-regulatory networks18. Deletion of the endoribonuclease Dicer19 or the microRNA miR-142 (ref. 20) leads to an increased proportion of B cells with an MZ phenotype, which suggests that microRNAs suppress the formation or survival of MZ B cells. Retroviral expression of the RBP lin28b in hematopoietic stem cells promotes the development of MZ B cells21,22, but roles have not been found for other RBPs in MZ B cells.

The ZFP36 family of RBPs are characterized by tandem CCCH-type zinc fingers, which bind RNA. By interacting with AU-rich elements in the 3′ untranslated region (UTR) of mRNA, these RBPs promote RNA decay23. ZFP36 has been best characterized as a suppressor of cytokine production in innate immune cells; its relatives ZFP36L1 and ZFP36L2 serve redundant roles during the development of T lymphocytes and B lymphocytes24,25. The function of the ZFP36 family during the development and maintenance of mature B lymphocytes has not been studied.

Here we found an indispensable role for ZFP36L1 in the maintenance of MZ B cells. Through analysis of the transcriptomes of primary mouse B cells and the identification of ZFP36L1 targets in B cells by individual-nucleotide-resolution cross-linking and immunoprecipitation (iCLIP), we identified the direct and indirect targets of ZFP36L1 in B cells and determined a network of factors under the
control of ZFP36L1 that promote the localization and survival of MZ B cells. Our study demonstrates that a single RBP can determine the cellular identity and, ultimately, the survival of MZ B cells.

RESULTS

MZ B cells specifically require ZFP36L1

To identify the roles of the ZFP36 family during lymphocyte development, we first generated Zfp36fl/flCD2-iCre- mice, in which loxP-flanked Zfp36 alleles (Zfp36fl/fl) are deleted by a transgene encoding ‘codon-improved’ Cre recombinase (iCre) expressed from the promoter of the human gene encoding the adhesion molecule CD2 (CD2) (Supplementary Fig. 1), as well as Zfp36fl/flCD2-iCre+ and Zfp36fl/flCD2-iCre- mice, in these mice, each RBP gene is deleted early in lymphoid ontogeny. CD21fl/flCD23- MZ B cells were ninefold less abundant in Zfp36fl/flCD2-iCre- mice than in Zfp36fl/flCD2-iCre+ mice but were normal in abundance in Zfp36fl/flCD2-iCre+ and Zfp36fl/flCD2-iCre- mice (Fig. 1a). The number of CD21fl/flCD23+ Fo B cells was slightly lower in Zfp36fl/flCD2-iCre+ mice than in Zfp36fl/flCD2-iCre- mice (Fig. 1b). Transcripts encoding the family members ZFP36, ZFP36L1 and ZFP36L2 were expressed in MZ B cells from C57Bl/6 mice (Fig. 1c), which indicated that the specific requirement for ZFP36L1 was not due to cell-specific mRNA expression. Thus, ZFP36L1 was required for the development or maintenance of MZ B cells, and its absence was not compensated for by ZFP36 or ZFP36L2.

B cell–intrinsic requirement for ZFP36L1

When Zfp36 was deleted at the pro-B cell stage in Zfp36fl/flMbICre+ mice (in which Zfp36l is deleted by Cre, expressed from a Cre-encoding gene knocked into the gene encoding the immunoglobulin α-chain signaling subunit Mb1 (CD79a)), these mice also showed a loss of MZ B cells relative to the abundance of these cells in their Zfp36fl/flMbICre+ littermates (data not shown). Zfp36fl/flCd23-Cre+ mice, in which Zfp36l is deleted at the transitional 2 (T2) B cell stage (by Cre expressed from a transgene derived from the gene encoding the low-affinity IgE receptor CD23), also had tenfold fewer MZ B cells and twofold fewer CD93+B220+IgM+CD21hi MZP B cells than did their Zfp36fl/flMbICre+ littermates (Fig. 2a,b). We observed a small but significant fourfold decrease in B220+CD19+ B-1 cells in the peritoneal cavity of Zfp36fl/flMbICre+ mice relative to their abundance in Zfp36fl/flMbICre+ mice, with both CD5+ B-1a cells and CD5− B-1b cells affected (Supplementary Fig. 2a–d). In contrast, the number of B220+CD19+tB2 cells in the peritoneal cavity of Zfp36fl/flMbICre+ mice was not different from that in Zfp36fl/flMbICre+ mice (Supplementary Fig. 2a,b). In contrast to the much larger effect of the deletion of ZFP36L1 on the number of MZ B cells, these observations suggested only a minor role for ZFP36L1 in B-1 cells.

To further understand the role of ZFP36L1 in mature B cells, we used CD23-Cre to express a fusion protein of green fluorescent protein (GFP) and ZFP36L1 (GFP–ZFP36L1) from the Rosa26 locus (R26GFPZFP36L1). In R26GFPZFP36L1Cd23-Cre+ mice, GFP–ZFP36L1 expression was observed from the T2 B cell stage (Supplementary Fig. 2e). The number of MZ B cells was 1.5-fold higher in R26GFPZFP36L1Cd23-Cre+ mice than in R26GFPZFP36L1Cd23-Cre- mice, but there were no effects on Fo B cells or MZP B cells (Fig. 2c,d). Immunofluorescence staining of spleen sections with antibodies to identify MZ B cells indicated that the MZ was fivefold smaller in Zfp36fl/flCd23-Cre+ mice and expanded by 1.5 fold in R26GFPZFP36L1Cd23-Cre+ mice relative to its size in Cre- control mice (Fig. 2e,f). This demonstrated a requirement for ZFP36L1 from the T2 B cell stage for the development and/or maintenance of MZ B cells.

Figure 1 MZ B cells specifically require ZFP36L1. (a.b) Quantification (by flow cytometry) of CD21fl/flCD23+ MZ B cells (a) and CD21fl/flCD23+ Fo (b) B cells in the spleen of Zfp36fl/flCD23-Cre+ or Zfp36fl/flCD23-Cre- mice (Zfp36fl/fl), Zfp36fl/flCD23-iCre- or Zfp36fl/flCD23-iCre+ mice (Zfp36fl/fl), and Zfp36fl/flCD23-iCre+ or Zfp36fl/flCD23-iCre- mice (Zfp36fl/fl) (key, CD23-Cre status). (c) qPCR analysis of Zfp36, Zfp36l1 and Zfp36l2 mRNA (horizontal axis) in sorted MZ B cells (CD21+CD23+) from C57Bl/6 mice; results are presented relative to those of the control mice and control mice (b) Zfp36l2mice (≥ 0.05 and **P < 0.01; Mann-Whitney). Data are from one experiment with n = 4 Zfp36fl/flCD23-Cre+ mice and n = 5 Zfp36fl/flCD23-Cre- mice (a,b), are pooled from two independent experiments with n = 7 (a) and 6 (b). Zfp36fl/flCD23-Cre+ mice, n = 8 (a) or 5 (b) Zfp36fl/flCD23-Cre- mice, n = 7 Zfp36fl/flCD23-Cre+ mice, and n = 5 (a) or 7 (b) Zfp36fl/flCD23-Cre- mice (a,b), or are from one experiment with n = 3 mice (c; mean + s.e.m.).

Maintenance of MZ and MZP B cells requires ZFP36L1

To investigate the continued requirement for ZFP36L1 in MZ B cells, we used a transgene encoding a fusion of Cre and the tamoxifen-inducible estrogen receptor variant ERT2, targeted to the mouse Rosa26 locus (R26ERT2-Cre). In this context, Cre activity is induced following the administration of tamoxifen to mice. In Zfp36fl/flR26ERT2-Cre+/mice, 7 d of tamoxifen treatment by oral gavage induced the recombination and deletion of Zfp36l1 in B cells and a 1.3-fold decrease in the number of MZ B cells relative to the number of these cells in Zfp36fl/+ R26ERT2-Cre+/mice (Supplementary Fig. 3a,b). The recombination efficiency of Zfp36l1 remained high at later time points, and MZ B cells decreased 1.7-fold by day 10 and 3.2-fold by day 14 of tamoxifen treatment in Zfp36fl/+R26ERT2-Cre+/mice relative to their abundance in Zfp36fl/+R26ERT2-Cre+ mice (Supplementary Fig. 3c–f). This ongoing deletion of MZ B cells was selective, as the number of Fo B cells was not lower at any of the three time points assessed (Supplementary Fig. 3b,d,f). To exclude the possibility of an effect of the deletion of ZFP36L1 in non-hematopoietic cells in Zfp36fl/flR26ERT2-Cre+/mice, we reconstituted lethally irradiated CD45.1+ B6.SJL mice with bone marrow from CD45.2+ Zfp36fl/flR26ERT2-Cre+ or CD45.2+ Zfp36l1+/+R26ERT2-Cre+ mice and, 8 weeks after reconstitution, administered tamoxifen to the host mice by oral gavage. At 7 d following tamoxifen treatment, purified B cells from Zfp36fl/flR26ERT2-Cre+ showed more than 80% deletion of Zfp36l1 (Fig. 3a). Chimeric mice reconstituted with Zfp36l1+/+R26ERT2-Cre+ bone marrow had a selective loss of MZ and MZP B cells at 7 d after tamoxifen treatment (Fig. 3b), while the
Figure 2  B cell–intrinsic requirement for ZFP36L1. (a) Flow cytometry of splenic B cells from Zfp36l1fl/flCd23-Cre+ and Zfp36l1fl/flCd23-Cre− mice (above plots; n = at least 9 mice per genotype), pre-gated on B220+CD93+ cells (top row) or B220+CD93− cells (bottom row). Numbers adjacent to outlined areas indicate percent cells in gate throughout. (b) Quantification (by flow cytometry) of CD21+CD23− MZ B cells (left), B220+CD93+CD21+IgM+ MZP B cells (right) and CD21+CD23− Fo B cells (middle) in the spleen of Zfp36l1fl/flCd23-Cre+ and Zfp36l1fl/flCd23-Cre− mice (key). (c) Flow cytometry (as in a) of splenic B cells from R26GFPZFP36L1Cd23-Cre− and R26GFPZFP36L1Cd23-Cre+ mice. (d) Quantification (as in b) of B cells from spleen of R26GFPZFP36L1Cd23-Cre− and R26GFPZFP36L1Cd23-Cre+ mice. (e) Quantification of MZ width in spleens from Zfp36l1fl/flCd23-Cre+ or Zfp36l1fl/flCd23-Cre− mice (Zfp36l1fl/fl and R26GFPZFP36L1Cd23-Cre− or R26GFPZFP36L1Cd23-Cre+ mice (R26GFPZFP36L1) (key, Cd23-Cre status), defined by CD1+ MZ B cells. (f) Immunofluorescence analysis of spleen sections from a Zfp36l1fl/fl control mouse (left), Zfp36l1fl/flCd23-Cre− mice (middle) and R26GFPZFP36L1Cd23-Cre+ mice (right), stained with anti-CD1d (magenta), anti-IgD (cyan) and anti-CD169 (MOMA-1; green) (top row) or anti-IgM (magenta) and anti-CD169 (MOMA-1; green) (bottom row); arrowheads indicate CD1d+IgD+ cells. Original magnification, ×10; scale bars, 100 μm. Each symbol (b,d) represents an individual mouse; small horizontal lines indicate the mean. **P ≤ 0.001 and ****P ≤ 0.0001 (Mann-Whitney). Data are representative of seven experiments (a), three experiments (c) or four experiments (f), pooled from three independent experiments representative of seven experiments with n = 9 mice (Zfp36l1fl/flCd23-Cre−) or n = 12 mice (Zfp36l1fl/flCd23-Cre−) (MZ B cells), n = 14 mice (Zfp36l1fl/flCd23-Cre−) or n = 13 mice (Zfp36l1fl/flCd23-Cre−) (Fo B cells), or n = 14 mice (Zfp36l1fl/flCd23-Cre− or Zfp36l1fl/flCd23-Cre+ (MZ B cells) (b) or pooled from two independent experiments representative of three experiments with n = 9 mice (R26GFPZFP36L1Cd23-Cre−) or n = 11 mice (R26GFPZFP36L1Cd23-Cre+) (MZ B cells), n = 9 mice (R26GFPZFP36L1Cd23-Cre−) or n = 11 mice (R26GFPZFP36L1Cd23-Cre+) (Fo B cells), or n = 9 mice (R26GFPZFP36L1Cd23-Cre− or R26GFPZFP36L1Cd23-Cre+) (MZ B cells) (d) mean) or with n = 51 measurements from 16 follicles (Zfp36l1fl/flCd23-Cre−), n = 58 measurements from 14 follicles (Zfp36l1fl/flCd23-Cre−), n = 41 measurements from 13 follicles (R26GFPZFP36L1Cd23-Cre+) or n = 55 measurements from 13 follicles (R26GFPZFP36L1Cd23-Cre−) (e; mean ± s.e.m.).

Fo and transitional B cell subsets remained unchanged in number relative to that in the chimeras reconstituted with Zfp36l1fl/flR26ERT2−Cre+ bone marrow (Fig. 3b and data not shown). Therefore, ZFP36L1 was dispensable for the maintenance of Fo B cells but was necessary for the persistence of MZ and MZP B cells.

To address whether ZFP36L1 affects B cell survival, we used flow cytometry to measure the presence of active caspase-3. There was a 2.5-fold greater proportion of MZ B cells positive for active caspase-3 in chimeras reconstituted with Zfp36l1fl/flR26ERT2−Cre+ bone marrow than in those reconstituted with Zfp36l1fl/flR26ERT2−Cre+ bone.
marrow (Fig. 3c). In contrast, there was no greater proportion of Fo B cells positive for active caspase-3 in chimeras reconstituted with Zfp36l1fl/flR26ERT2−Cre/+ than in chimeras reconstituted with Zfp36l1fl/flR26ERT2−Cre/+ bone marrow (Fig. 3c). Furthermore, the proportion of cells positive for active caspase-3 was similarly greater among MZ B cells (but not Fo B cells) from Zfp36l1fl/flMb1Cre/+ mice than among those from Zfp36l1fl/flMb1Cre/+ mice (Fig. 3d).

We assessed the turnover of MZ B cells in Zfp36l1fl/flMb1Cre/+ mice by measuring labeling with the thymidine analog BrdU following its administration for 14 d in the drinking water. BrdU is incorporated into highly proliferative pre-B cells in the bone marrow but not into non-proliferative transitional or naive B cell subsets, including MZ B cells.29 At day 14, 50% of MZ B cells were BrdU+ in Zfp36l1fl/flMb1Cre/+ mice, but only 30% of MZ B cells were BrdU+ in Zfp36l1fl/flMb1Cre/+ mice (Fig. 3e). Approximately 17% of Fo B cells were BrdU+ in both Zfp36l1fl/flMb1Cre/+ mice and Zfp36l1fl/flMb1Cre/+ mice (Supplementary Fig. 4a). There were slightly fewer BrdU+ T2 and MZP B cells in Zfp36l1fl/flMb1Cre/+ mice than in Zfp36l1fl/flMb1Cre/+ mice (Supplementary Fig. 4a).

In contrast, BrdU incorporation was 1.5-fold lower in MZ B cells and slightly greater in the T2 B cells of in R26GFPPZF36L1Cd23-Cre− mice than in those of R26GFPPZF36L1Cd23-Cre− mice (Fig. 3f and Supplementary Fig. 4b). These data indicated that ZFP36L1 was not required for the survival of Fo B cells but was essential for the survival of MZ B cells.

**Gene-expression changes after inducible deletion of Zfp36l1**

ZFP36L1 controls gene expression by promoting RNA decay23,25. To identify direct targets of ZFP36L1, we performed RNA-based next-generation sequencing (RNA-seq) of sorted MZ B cells from tamoxifen-treated Zfp36l1fl/flR26ERT2−Cre−/+ and Zfp36l1fl/flR26ERT2−Cre−/+ bone marrow (key) and treated with tamoxifen; results are presented relative to those of the control gene Tbp. (b) Quantification (by flow cytometry) of CD21+CD23+ MZ B cells, CD21+IgM+ MZP B cells and CD21+CD23+ Fo B cells in the spleen of chimeras as in a. (c,d) Frequency (assessed by flow cytometry) of MZ and Fo B cells (as in b) that stained positive for active caspase-3 (Casp3+) among cells from the spleen of chimeras as in a (c) or Zfp36l1fl/flMb1Cre/+ and Zfp36l1fl/flMb1Cre− mice (d). (e,f) Incorporation of BrdU into MZ B cells (as in b) the spleen of Zfp36l1fl/flMb1Cre+ and Zfp36l1fl/flMb1Cre− mice (e) or R26GFPPZF36L1Cd23-Cre− and R26GFPPZF36L1Cd23-Cre− mice (f), assessed by flow cytometry at day 14 of BrdU treatment. Each symbol (b-f) represents an individual mouse; small horizontal lines indicate the mean. ** P ≤ 0.01 and *** P ≤ 0.001 (Mann-Whitney).

Gene-expression changes after inducible deletion in Zfp36l1

We observed a significantly lower number of reads mapped within the loxP-flanked region of Zfp36l1 in MZ B cells from Zfp36l1fl/flR26ERT2−Cre−/+ than in that region in similar cells from Zfp36l1fl/flR26ERT2−Cre−/+ mice (Supplementary Fig. 5a), which indicated effective deletion of Zfp36l1. After loss of Zfp36l1, we observed a significantly higher expression of 330 transcripts and lower expression of 215 transcripts in Zfp36l1fl/flR26ERT2−Cre−/+ MZ B cells than in Zfp36l1fl/flR26ERT2−Cre−/+ MZ B cells. Of those, the expression of 84 transcripts and 26 transcripts was increased or decreased, respectively, by more than 1.5-fold (Fig. 4a and Supplementary Tables 1–3). We also observed an increase in the expression of Zfp36l2 after deletion of Zfp36l1 (Fig. 4a, Supplementary Fig. 5b), which suggested that ZFP36L2 did not fully functionally compensate for ZFP36L1 in MZ B cells.

iCLIP can identify the direct targets of RBPs and the specific nucleotide contacts between RBPs and RNA, but this method requires a large number of cells and is not sensitive enough to apply to the small number of MZ B cells available. Therefore, we used iCLIP data for ZFP36L1 in activated Fo B cells25 to identify candidate miRNAs that can be bound by ZFP36L1. 73 genes with higher expression in Zfp36l1fl/flR26ERT2−Cre−/+ MZ B cells than in Zfp36l1fl/flR26ERT2−Cre−/+ MZ B cells, 11 of which showed a change in expression of over 1.5-fold, were identified by iCLIP as possible direct targets of ZFP36L1 (Fig. 4b and Supplementary Tables 2 and 3). 24 transcripts with lower expression in Zfp36l1fl/flR26ERT2−Cre−/+ MZ B cells than in Zfp36l1fl/flR26ERT2−Cre−/+ MZ B cells were also found in the iCLIP data for ZFP36L1; however, only one transcript, Per2 (which encodes the circadian protein mPer2), exhibited a change in expression of over 1.5-fold (Fig. 4b and Supplementary Table 2), which suggested that
our GSEA analysis using the software tool REVIGO to reduce and visualize gene ontology. This analysis indicated that many of the differentially expressed transcripts encoded products involved in signaling, cellular adhesion and migration, cell cycle and proliferation, and programmed cell death (Fig. 4c). Transcripts with lower expression did not encode products that constitute any common pathway or gene set, which suggested that these transcripts did not have a common function in MZ B cells.

The ZFP36 family of RBPs has been shown to promote cell quiescence\textsuperscript{25,26}. The expression of Ccn2 mRNA (which encodes cyclin A2), Cdc6 mRNA (which encodes the cell-division protein CDC18L), Pim1 mRNA (which encodes the serine-threonine kinase PIM1), and Cdki mRNA (which encodes the cyclin-dependent kinase Cdc2) was ≥1.5-fold higher in Zfp36l1\textsuperscript{fl/fl}R26\textsuperscript{ERT2-Cre/+} MZ B cells than in Zfp36l1\textsuperscript{fl/fl}R26\textsuperscript{ERT2-Cre/+} MZ B cells (Supplementary Tables 1 and 2). To assess the consequences of that, we analyzed the proportion of MZ B cells in Zfp36l1-deficient mice expressing the cyclin-dependent kinase inhibitor p27\textsuperscript{KIP1} (encoded by Cdkn1b), a marker of cell quiescence. The expression of p27 in MZ B cells and the proportion of p27\textsuperscript{+} MZ B cells isolated from tamoxifen-treated Zfp36l1\textsuperscript{fl/fl}R26\textsuperscript{ERT2-Cre/+} mice was similar to that in Zfp36l1\textsuperscript{fl/fl}R26\textsuperscript{ERT2-Cre/+} mice (Supplementary Fig. 6a-c). The expression of p27 was unchanged in MZ B cells from Zfp36l1\textsuperscript{fl/fl}ERT2\textsuperscript{Cre/+} mice relative to its expression in such cells from Zfp36l1\textsuperscript{fl/fl}R26\textsuperscript{ERT2-Cre/+} mice (Supplementary Fig. 6a-c). Furthermore, the frequency of MZ B cells that stained positive for the proliferation marker Ki67 in Zfp36l1\textsuperscript{fl/fl}R26\textsuperscript{ERT2-Cre/+} mice was not different the staining of such cells in MZ B cells from Zfp36l1\textsuperscript{fl/fl}R26\textsuperscript{ERT2-Cre/+} mice, and the proportion of MZ B cells that stained with the DNA-binding dye DAPI was also unchanged in these mice (Supplementary Fig. 6d-g).

Together these data suggested that the reduction in the number of MZ B cells after developmentally programmed or induced deletion of Zfp36l1 was not due to loss of quiescence.

**ZFP36L1 enforces MZ B cell identity**

To further understand the changes in the MZ B cell transcriptome that arose from deletion of Zfp36l1, we compared transcripts that were expressed differentially in Zfp36l1\textsuperscript{fl/fl}R26\textsuperscript{ERT2-Cre/+} MZ B cells relative to their expression in Zfp36l1\textsuperscript{fl/fl}R26\textsuperscript{ERT2-Cre/+} MZ B cells with transcripts that were expressed differentially in Zfp36l1\textsuperscript{fl/fl}R26\textsuperscript{ERT2-Cre/+} (wild-type) MZ B cells relative to their expression in Zfp36l1\textsuperscript{fl/fl}R26\textsuperscript{ERT2-Cre/+} (wild-type) MZ B cells. 72% (54) of the transcripts with the greatest increase in expression in Zfp36l1\textsuperscript{fl/fl}R26\textsuperscript{ERT2-Cre/+} MZ B cells relative to that in Zfp36l1\textsuperscript{fl/fl}R26\textsuperscript{ERT2-Cre/+} MZ B cells had higher expression in wild-type MZ B cells than in wild-type MZ B cells (Fig. 5a and Supplementary Table 4). Only a subset of the transcripts for which Fo B cells showed enrichment (relative to their abundance in MZ B cells) were increased in Zfp36l1\textsuperscript{fl/fl}R26\textsuperscript{ERT2-Cre/+} MZ B cells (Fig. 5a), which indicated that the sorted Zfp36l1\textsuperscript{fl/fl}R26\textsuperscript{ERT2-Cre/+} MZ B cells were not contaminated with Zfp36l1\textsuperscript{fl/fl}R26\textsuperscript{ERT2-Cre/+} Fo B cells. Some transcripts that had higher expression in MZ B cells than in Fo B cells in wild-type mice, such as Plxnd1 (which encodes plexin D1) and Myc (which encodes the cell-cycle regulator c-Myc), also had higher expression in Zfp36l1\textsuperscript{fl/fl}R26\textsuperscript{ERT2-Cre/+} MZ B cells after treatment with tamoxifen had higher expression of transcripts associated with a Fo B cell phenotype.

To determine the effect of GFP–ZFP36L1 on the transcriptome of Fo B cells, we used RNA-seq analysis to compare the transcriptome of Fo B cells sorted from R26\textsuperscript{GFPZFP36L1}Cd23\textsuperscript{Cre−/+} mice with that of Fo B cells from R26\textsuperscript{GFPZFP36L1}Cd23\textsuperscript{Cre−/+} mice. Pairwise...
comparison with genes expressed differentially by wild-type Fo B cells relative to their expression by MZ B cells indicated that the Fo B cells from R26GFP/Zfp36l1-lox/loxCd23-Cre+ mice showed a trend toward higher expression of genes 'preferentially' expressed by MZ B cells and lower expression of genes characteristically expressed by Fo B cells (Fig. 5b and Supplementary Table 5). A negative correlation was evident for genes expressed differentially by Fo B cells from R26GFP/Zfp36l1-lox/loxCd23-Cre+ mice relative to their expression in Fo B cells from R26GFP/Zfp36l1-lox/loxCd23-Cre+ mice and genes expressed differentially by Zfp36l1-lox/loxCd23-Cre+ MZ B cells relative to their expression in Zfp36l1-lox/loxFoB cells (Fig. 5c and Supplementary Table 6), which suggested that ZFP36L1 was needed to maintain the identity of MZ B cells.

We next assessed cell-surface markers expressed by Fo B cells from R26GFP/Zfp36l1-lox/loxCd23-Cre+ mice. GFP+ Fo B cells from those mice had higher expression of CD21, CD1d and major histocompatibility complex class II than that of Fo B cells from R26GFP/Zfp36l1-lox/loxCd23-Cre+ mice (Fig. 5d), indicative of a phenotype and 'activation' status that resembled that of MZ B cells. Furthermore, staining of spleen sections with fluorescent antibodies showed a greater abundance of CD1d+ cells that also expressed IgD within the follicles of R26GFP/Zfp36l1-lox/loxCd23-Cre+ mice than in those of R26GFP/Zfp36l1-lox/loxCd23-Cre+ mice (Fig. 5f).

MZ B cells show enhanced calcium flux elicited by the B cell receptor (BCR) compared with that of Fo B cells81. Because a few of the transcripts expressed differentially by Zfp36l1-lox/loxFoB cells relative to their expression in Zfp36l1-lox/loxFoB cells encode products with roles in cell signaling, we measured calcium flux elicited by cross-linkage of surface IgM. It was observed that BCR-elicted calcium flux was enhanced in MZ B cells relative to that in Fo B cells in control Zfp36l1-lox/loxB6.1/Mb1+/+ mice (Fig. 5e), while calcium flux in MZ B cells from Zfp36l1-lox/loxB6.1/Mb1Cre+ was similar to
that observed in Fo B cells from those mice (Fig. 5e). Calcium flux elicited by cross-linkage of the BCR in CD93+ transitional B cell subsets (data not shown) or CD21+CD23- Fo B cells (Fig. 5e) from Zfp36l1fl/flMb1Cre/+ mice was not different from that elicited in the same subsets from Zfp36l1fl/flMb1+/+ mice.

Incubation of cells with EGTA chelates extracellular calcium and limits BCR-stimulated calcium flux to that released from internal stores. Stimulation with antibody to IgM (anti-IgM) elicited less calcium release in EGTA-treated MZ B cells from Zfp36l1fl/flMb1Cre/+ mice than in those from Zfp36l1fl/flMb1+/+ mice (Fig. 5e). This indicated defective release of calcium from the internal stores of Zfp36l1fl/flMb1Cre/+ MZ B cells. Thus, ZFP36L1 enhanced BCR signaling in MZ B cells.

To investigate the function of the MZ B cells in the ZFP36L1-deficient mice that remained after deletion of Zfp36l1, we immunized Zfp36l1fl/flCd23-Cre- and R26GFPZFP36L1Cd23-Cre- mice, as well as their control littermates (Zfp36l1fl/flCd23-Cre- and R26GFPZFP36L1Cd23-Cre- respectively), with the CD1d-restricted antigen NP-α-GalCer3,4. 4 d after immunization, the titer of NP-specific IgM in serum was similar in Zfp36l1fl/flCd23-Cre- mice and Zfp36l1fl/flCd23-Cre- mice (Supplementary Fig. 7b), which indicated that the number of MZ B cells in ZFP36L1-deficient mice was not limiting the antibody response to antigen in vivo. These data suggested that ZFP36L1 did not have a role in the MZ B cell response to CD1d-restricted humoral responses in vivo but enforced the MZ B cell phenotype in mice.

ZFP36L1 targets IRF8 and KLF2 to enforce MZ B cell identity

Loss of IRF8 leads to an enlarged MZ B cell compartment33, suggestive of a role for IRF8 in limiting the number of MZ B cells. The expression of IRF8 mRNA was 1.3-fold higher in MZ B cells from Zfp36l1fl/fl R26ERT2-Cre/+ than in those from Zfp36l1fl/flR26ERT2-Cre/T2-Cre- mice (Fig. 6a) and was lower in in Fo B cells from R26GFPZFP36L1Cd23-Cre+ mice than in those from R26GFPZFP36L1Cd23-Cre- mice (Fig. 6b). The expression of IRF8 protein was also higher in MZ B cells from Zfp36l1fl/flMb1Cre/+ than in those from Zfp36l1fl/flMb1+/+ mice (Fig. 6c,d). IRF8 mRNA contains a highly conserved AU-rich element in its 3′ UTR and was bound by ZFP36L1 in our iCLIP analysis of activated MZ B cells (Fig. 6e), which indicated it was probably a direct target of ZFP36L1 in MZ B cells.

To determine whether genes that are targets of IRF8 contributed to the loss of MZ B cells in the absence of ZFP36L1, we sought to determine whether transcripts that were expressed differentially by Zfp36l1fl/fl R26ERT2-Cre/+ MZ B cells relative to their expression in Zfp36l1fl/fl R26ERT2-Cre/T2-Cre- MZ B cells were identified as targets of IRF8 in high-quality data obtained by chromatin immunoprecipitation followed by deep sequencing (ChIP-seq)33. We found that a subset of the transcripts for which wild-type Fo B cells showed enrichment relative to their abundance in wild-type MZ B cells and that had higher expression in Zfp36l1fl/fl R26ERT2-Cre/+ MZ B cells than in Zfp36l1fl/fl R26ERT2-Cre/T2-Cre- MZ B cells were bound by IRF8 (Fig. 6f).
This suggested that higher expression of IRF8 contributed directly to the altered expression of these genes in the absence of ZFP36L1. Furthermore, the distribution of direct ZFP36L1 targets identified by iCLIP analysis showed minimal overlap with IRF8 targets (Fig. 6f,g).

Figure 7 ZFP36L1 regulates KLF2. (a) Expression of Klf2 mRNA (normalized read counts) in CD21<sup>+</sup>CD23<sup>−</sup> MZ B cells from tamoxifen-treated Zfp36l1<sup>fl/fl</sup>R26<i>ERT2−Cre</i>/ERT2−Cre and Zfp36l1<sup>fl/fl</sup>R26<i>ERT2−Cre</i>/+ mice (key). (b) Flow cytometry analyzing KLF2 in MZ B cells in Zfp36l1<sup>fl/fl</sup>Mb1<sup>Cre</sup> and Zfp36l1<sup>fl/fl</sup>Mb1<sup>Cre+</sup> mice (key). Isotype, isotype-matched control antibody. (c) Summary of flow cytometry analyzing KLF2 in MZ B cells from Zfp36l1<sup>fl/fl</sup>R26<i>ERT2−Cre</i>/+ and Zfp36l1<sup>fl/fl</sup>R26<i>ERT2−Cre</i>/− mice (key). Each symbol represents an individual mouse; small horizontal lines indicate the mean.

The expression of Klf2 mRNA was 3.1-fold higher in Zfp36l1<sup>fl/fl</sup>R26<i>ERT2−Cre</i>/+ MZ B cells than in Zfp36l1<sup>fl/fl</sup>R26<i>ERT2−Cre</i>/− MZ B cells (Fig. 7a). The expression of KLF2 protein was also higher, as assessed by flow cytometry, in Zfp36l1<sup>fl/fl</sup>Mb1<sup>Cre+</sup> MZ B cells than in Zfp36l1<sup>fl/fl</sup>Mb1<sup>Cre</sup> MZ B cells (Fig. 7b) and also in Zfp36l1<sup>fl/fl</sup>R26<i>ERT2−Cre</i>/+ MZ B cells than in Zfp36l1<sup>fl/fl</sup>R26<i>ERT2−Cre</i>/− MZ B cells (Fig. 7c). Klf2 mRNA contains a TATTATT AU-rich element in its 3′ UTR that is conserved among mammalian species that have an ortholog of this mRNA (Fig. 7d). iCLIP analysis indicated that ZFP36L1 bound in this AU-rich element (Fig. 7d); however, the data did not reach statistical significance due to the low abundance of Klf2 mRNA in activated B cells<sup>15,34</sup>. Thus, ZFP36L1 might directly limit expression of KLF2.

To determine if KLF2 contributed to the altered gene-expression profile of Zfp36l1<sup>fl/fl</sup>R26<i>ERT2−Cre</i>/+ MZ B cells, we used ChIP-seq data for KLF2 (ref. 35) and microarray analysis of transcripts expressed differentially by Klf2<sup>−/−</sup>Cd19<sup>−</sup>Cre<sup>+</sup> B cells relative to their expression by wild-type B cells<sup>14</sup> to generate a list of candidate targets of KLF2 relevant to B cells. This list identified a set of genes that were bound by KLF2 and had higher expression in Zfp36l1<sup>fl/fl</sup>R26<i>ERT2−Cre</i>/+ MZ B cells than in Zfp36l1<sup>fl/fl</sup>R26<i>ERT2−Cre</i>/− MZ B cells and higher expression in wild-type Fo B cells than in wild-type MZ B cells (Fig. 7e). Furthermore, this analysis showed that among the transcripts with higher expression in Zfp36l1<sup>fl/fl</sup>R26<i>ERT2−Cre</i>/+ MZ B cells, the KLF2-bound genes were generally absent from both the iCLIP data set for ZFP36L1 and the ChIP-seq data set for IRF8 (Fig. 7f), which indicated that the overexpression of KLF2 in Zfp36l1<sup>fl/fl</sup>R26<i>ERT2−Cre</i>/+ MZ B cells was consequential for the transcriptome. Together these analyses suggested a model whereby ZFP36L1 limits the expression of a few genes, including those encoding the transcription factors IRF8 and KLF2, which in turn regulate genes important for the identity and survival of MZ B cells (Supplementary Fig. 7c).

ZFP36L1 regulates B cell localization

GSEA of 116 transcripts that had both higher expression in Zfp36l1<sup>fl/fl</sup>R26<i>ERT2−Cre</i>/+ MZ B cells and lower expression in Fo B cells from Zfp36l1<sup>fl/fl</sup>R26<i>ERT2−Cre</i>/− mice than in those from Zfp36l1<sup>fl/fl</sup>R26<i>ERT2−Cre</i>/+ mice (Supplementary Fig. 8a) identified pathways involved in B cell trafficking (Supplementary Fig. 8b). Flow cytometry indicated higher expression of the adhesion molecule CD62L and the integrin β<sub>7</sub>, but no difference in the expression of the C-type lectin CD69 (a surrogate marker for the receptor S1PR1), on the surface of Zfp36l1<sup>fl/fl</sup>Mb1<sup>Cre</sup> MZ B cells relative to their expression on the surface of Zfp36l1<sup>fl/fl</sup>Mb1<sup>Cre+</sup> MZ B cells, and lower expression of CD62L and β<sub>7</sub> on Fo
which indicated that the greater MFI of CD1d in MZ B cells and CD21-cre mice (GFP–ZFP36L1) (genotypes in keys). (b) Flow cytometry (left) analyzing the binding of PE-conjugated anti-CD19 (CD19–PE) by splenic MZ B cells from Zfp36l1+/+Cd23-Cre+ and Zfp36l1fl/fl mice (key), and frequency of CD19–PE+ splenic MZ B cells (right) in mice as at left. Each symbol (right) represents an individual mouse; small horizontal lines indicate the mean. (c) Immunofluorescence microscopy of splenic sections from tamoxifen-treated Zfp36l1fl/flR26ERT2-Cre/+ μMT chimeras (left) and Zfp36l1fl/flR26ERT2-Cre/+ μMT chimeras (right), stained with anti-CD1d (magenta), anti-IgD (cyan) and anti-CD1d (MOMA-1; green). Original magnification, x20; scale bars, 50 μm. (d) MFI of CD1d in the MZ area (defined by CD1d staining outside of staining for MOMA-1 and IgD; left) and in follicles (defined by MOMA-1 staining; middle), and MFI in follicles relative to total MFI (MZ plus Fo (normalized values); right); all results are calculated as CD1d MFI – background. ** P < 0.01 (Mann-Whitney). Data are representative of one experiment with five mice per genotype (a), pooled from two experiments with n = 5 mice (b) or are from one experiment (c,d); mean ± s.e.m. of 10 follicles (Zfp36l1+/+R26ERT2-Cre-/+μMT) or 25 follicles (Zfp36l1fl/flR26ERT2-Cre-/+μMT) in (d).

B cells from R26GFPZFP36L1Cd23-Cre+ mice than on such cells from R26GFPZFP36L1Cd23-Cre+ mice (Fig. 8a). We observed little if any difference in expression of the chemokine receptors CXCR4 and CXCR5, the integrins β1 and LFA-1 (αLβ2) on the surface of MZ and Fo B cells from Zfp36l1fl/flCd23-Cre+ mice relative to that on such cells from Zfp36l1fl/flCd23-Cre+ mice (data not shown). The expression of mRNA encoding S1PR1 in MZ B cells from Zfp36l1fl/flR26ERT2-Cre+ was also not different from that in MZ B cells from Zfp36l1+/+R26ERT2-Cre-/+ mice (Supplementary Fig. 8c). To assess whether ZFP36L1 affected the localization of MZ B cells, we measured by flow cytometry the binding of phycoerythrin–conjugated anti-CD19 to MZ B cells, following intravenous administration to mice. The binding of this antibody to MZ B cells from Zfp36l1fl/flCd23-Cre+ cells was 1.5-fold lower than its binding to MZ B cells from Zfp36l1+/+Cd23-Cre+ mice (Fig. 8b), which indicated that ZFP36L1 promoted localization to the MZ.

We generated mixed-bone-marrow chimeras by reconstituting lethally irradiated B6.129J mice with bone marrow from Zfp36l1+/+/R26ERT2-Cre+ or Zfp36l1fl/flR26ERT2-Cre+ mice, together with bone marrow from μMT mice (which lack mature B cells); this resulted in ‘Zfp36l1+/+R26ERT2-Cre+’μMT chimeras and ‘Zfp36l1fl/flR26ERT2-Cre+’μMT chimeras. Following reconstitution, we administered tamoxifen to induce deletion of Zfp36l1 and measured the localization of CD1d+ cells by antibody staining of splenic tissue sections. We observed a greater proportion of CD1d+ B cells in the splenic follicles of Zfp36l1+/+R26ERT2-Cre+μMT chimeras than in those of Zfp36l1fl/flR26ERT2-Cre-/+μMT chimeras (Fig. 8c). CD1d+ B cells in the splenic follicles of Zfp36l1+/+R26ERT2-Cre+μMT chimeras did not express IgD (Supplementary Fig. 7a), which indicated that they were MZ B cells. We defined the boundaries of the splenic follicle by staining of MOMA-1, a marker specific for metallophilic macrophages (Supplementary Fig. 8e), and quantified the mean fluorescent intensity (MFI) of CD1d within the follicle. The MFI of CD1d was greater in the splenic follicles of Zfp36l1+/+R26ERT2-Cre+μMT chimeras than in those of Zfp36l1fl/flR26ERT2-Cre-/+μMT chimeras (Fig. 8d).

The MFI of CD1d in the MZ of Zfp36l1+/+R26ERT2-Cre+μMT chimeras was not greater than that in the MZ of Zfp36l1+/+R26ERT2-Cre+μMT chimeras (Fig. 8d), which indicated that the greater MFI of CD1d in
the follicles of Zfp36l1fl/flR26ERT2−Cre+/µMT chimeras did not result from an increase in CD1d expression on all B cells. Normalization of the MFI of CD1d within the splenic follicle to the MFI of CD1d within the Fo and MZ confirmed this result (Fig. 8d). Thus, ZFP36L1 was required for the proper localization of MZ B cells.

DISCUSSION

Here we reported an essential role for ZFP36L1 in the maintenance of MZ B cells. ZFP36L1 mediated this role by interacting with and limiting the expression of a set of transcripts that promoted the Fo B cell phenotype. The relevant targets of ZFP36L1 in this context were distinct from previously identified targets of ZFP36L1 and included genes encoding the transcription factors IRF8 and KLF2 and regulators of adhesion and migration.

This ability of ZFP36L1 to regulate different transcripts at discrete stages of development is reminiscent of transcription factors that control gene expression characteristic of a particular developmental stage. Regulation by ZFP36L1 can be considered a post-transcriptional RNA operon or ‘regulon’ that maintains the identity of MZ B cells. ZFP36L1-related proteins regulate cell fate in other developmental biology systems. In Caenorhabditis elegans, the germline and somatic cell fates are regulated by multiple RBPs, many of which contain tandem CCCH zinc fingers. Among those, OMA-1 (ref. 38) and POS-1 (ref. 39) bind with high affinity to AU-rich sequences in the 3′ UTR of mRNA. Systems analysis has indicated extensive crosstalk between RBPs and transcription factors in C. elegans. Our findings have indicated the existence of similar networks in mammalian cells. Establishing this principle in additional mammalian systems will require the identification of both the relevant RBPs and the bound targets.

The specific requirement for ZFP36L1 in MZ B cells is in contrast to the redundant function of ZFP36L1 and ZFP36L2 in early lymphocyte development. ZFP36L1 binds to Zfp36l2 mRNA, and the abundance of Zfp36l2 mRNA was increased in Zfp36l1fl/flR26ERT2−Cre+/MZ B cells, which suggested that ZFP36L1 suppressed its paralog in MZ B cells. It thus appears that ZFP36L2 was unable to compensate for the absence of ZFP36L1 in MZ B cells. This might reflect differences in the post-translational biology of the RBPs encoded, such as the effects of specific phosphorylation or of multi-protein complex formation. Alternatively, there may be differences among members of the ZFP36 family in their ability to bind to and regulate specific targets. Extensive further work will be needed to elucidate the molecular basis of the redundant and non-redundant functions of these RBPs.

We identified IRF8 and KLF2 as direct targets of ZFP36L1 that regulated many genes encoding products important for MZ B cell identity. The molecular basis for the regulation of the MZ B cell pool by KLF2 might also relate to its ability to control the expression of adhesion receptors, while the mechanisms by which IRF8 limits the size of the MZ B cell compartment is unclear. Many, but not all, of the indirect changes in the transcriptome of Zfp36l1fl/flR26ERT2−Cre+/MZ B cells appeared to stem from their higher expression of IRF8 or KLF2, as these transcription factors accounted for 18 of 54 differentially expressed ‘signature’ genes of Fo B cells. Many targets of KLF2 that were not targets of ZFP36L1 had higher expression in Zfp36l1fl/flR26ERT2−Cre+/MZ B cells, and many of these were also suppressed in Fo B cells from R26GFPZFP36L1Cd23Cre+ mice. Thus, the transcriptome of MZ B cells was regulated by a network of factors that acted transcriptionally and post-transcriptionally, in which ZFP36L1 was a major hub. Additional targets of ZFP36L1 identified by iCLIP analysis with higher expression in Zfp36l1fl/flR26ERT2−Cre+/MZ B cells encoded products that might contribute to the abnormal localization and survival of MZ B cells. The following have all been linked to adhesion and metastasis: PIM1, which is a kinase; KIAA0101 (2810417H13Rik), a factor associated with proliferating cell nuclear antigen; Myadm (‘myeloid-associated differentiation marker’), a putative adaptor of uncertain function; Txnrd1 (‘thioredoxin reductase 1’), an enzyme that catalyzes the reduction of thioredoxin; and Bhlhe40, a transcriptional regulator. ZFP36L1 might act to promote the interaction between MZ B cells and the unique extracellular matrix components for which the MZ is enriched and might have an important role in promoting the survival of MZ B cells. Such mechanisms protect MZ B cells from apoptosis. We are therefore tempted to speculate that ZFP36L1-deficient MZ B cells lack such pro-survival signals when access to the MZ is limited. This might be analogous to the process of anoikis, whereby detachment of adherent cells from a niche can lead to programmed cell death. In summary, our data suggest that ZFP36L1 acts post-transcriptionally to enforce the phenotype of MZ B cells and that in its absence, MZ B cells are mislocalized and die. It will be important for future studies to establish whether these mechanisms contribute to pathology in lymphoma or autoimmune disease.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

R.N. designed and did most experiments; H.A. performed bioinformatics analysis; A.S. performed iCLIP analysis; A.G., D.I.H., R.W. and S.E.B. helped with mouse experiments; G.S.B. provided NP-α-GaLCer; C.N.C. and A.F.C. performed some histology; and R.N. and M.T. planned the project and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Reagents, antibodies and oligonucleotides. This information is provided in Supplementary Tables 7 (reagents), 8 (antibodies) and 9 (oligonucleotides).

Mouse strains and animal procedures. Mice on the C57BL/6 background used in this study were derivatives of the following: Tg(CD2-cre)4Kio mice51, CD79atm1(Tnr)B10 mice52, Tg(Fcεr2a-cre)3SMu mice26, Gt(Rosa)26Sor1(CreERT2)1Thi mice53, Zfp36tm1Thi mice (Supplementary Fig. 1), Zfp36tm1.1Thi mice24, Zfp36tm1.1Thi mice24, Gt(Rosa)26Sor1(GFPtsp5361)Thi mice25 and B6.129S1-Bcl21tm11Lav mice54. All mice were between 8 and 12 weeks of age. B6.SJL mice, which were used in bone marrow chimera experiments, are Ly5.1 (C57BL/6 allotype) C57BL/6 congenic mice obtained from Jackson labs, USA. For bone-marrow chimeras, B6.SJL recipient mice were irradiated and reconstituted with a total of 3 x 10⁶ donor bone marrow cells by intravenous (i.v.) injection. Reconstituted mice were fed neomycin sulfate (Sigma) in their drinking water for four weeks post-reconstitution, and were analyzed after 8-10 weeks. In the case of μMT chimeras, of the 3 x 10⁶ donor BM cells, 80% were obtained from μMT mice and 20% were obtained from either Zfp36tm11/1R26R2ERT2-Cre; or Zfp36tm11/1R26R2ERT2-Cre− control mice. BrdU (Sigma) was administered at 0.8 mg/ml in drinking water. Unless otherwise stated, control mice used in experiments were control littersmates that were negative for Cre. Tomoxifen (Cambridge Bioscience Ltd.) was prepared in sunflower oil containing 10% ethanol to a final concentration of 50 mg/ml. Daily dosage for mice was 200 mg/kg. Tomoxifen was fed to mice for 2 consecutive days using a bulb-tipped reusable feeding needle. After induction of Cre, mice were returned to stock and euthanized at the appropriate time point. 0.8 μg of PE-labeled anti-mouse CD19 (Supplementary Table 8) in PBS was administered intravenously and mice were culled at 5 min following injection. Spleen cell suspensions were prepared and surface stained as described. The Animal Welfare and Experimentation Committee of the Babraham Institute and the UK Home Office approved all animal procedures at the Babraham Institute.

Immunizations and ELISA. Mice were immunized intravenously with 1 μg/ml NP-OvalCer22 in 200 μl PBS containing 0.05% BSA that had been sonicated. Serum was prepared from blood collected at day 4 after immunization. Relative endpoint titers for serum antibody were determined by ELISA. For determination of NP-specific antibodies, ELISA plates (Nunc MaxiSorp) were coated with NP(23)-BSA (Sigma, Biosearch Technologies) and were blocked with 1% BSA in PBS. Serial dilutions of serum samples (0.1% BSA/PBS) were added and incubated overnight. Bound IgM-specific antibodies were detected with biotinylated anti-mouse IgM-specific immunoglobulin (Southern Biotech) followed by streptavidin HRP (Southern Biotech) and developed with Sigma Fast O-phenylenediamine dihydrochloride (Sigma-Aldrich) as a substrate, and the absorbance at 490 nm was determined. Absorbance values were plotted from serially diluted samples and values, which fell into the linear range of the curve were used to calculate endpoint titers.

Flow cytometry. B cell populations were analyzed using antibodies to B220, Cd19, Cd21, Cd23, Cd93, IgD and IgM (Supplementary Table 8). B cells analyzed in the bone marrow were as follows: immature B cells, B220+IgM+IgD−; recirculating B cells, B220+IgM+IgD+. B cells analyzed in the spleen were as follows: T1 B cells, B220+CD93+CD23+IgM+; T2 B cells, B220+CD93+CD23+IgM−; T3 B cells, B220+CD93+CD23+IgM−; MZPs, B220+CD93+CD21+IgM+; MZ B cells, B220+CD93+CD21+CD23− or B220+CD9+CD123+; and Fo B cells, B220+CD9+CD21+CD23−. B cells analyzed in the peritoneal cavity were as follows: B-1 cells, B220+CD19+; B-2 cells, B220+CD19−; B-1a cells, B220+CD19+CD5+IgM−; and B-1b cells, B220+CD19+CD5+IgM+. Fixable viability dye eFluor780 was used to assess cell viability. BrdU was detected using the BrdU flow kit (BD), following the manufacturer’s instructions. Cells were analyzed using a BD LSRFortessa flow cytometer. To measure apoptosis, 5 x 10⁶ cells were stained intracellularly with a FITC-conjugated rabbit monoclonal antibody that recognizes active caspase-3 (Supplementary Table 8; BD Biosciences).

Calcium flux analysis. Splenocytes were loaded for 30 min at 37°C in the dark with 0.6 μM Ca²⁺ indicator PRX (BD) in serum free DMEM (5 x 10⁶ to 10 x 10⁶ cells). Cells were then stained with surface antibodies at RT and were resuspended in serum free Hanks medium. Cells were pre-treated with 10 mM EGTA for 1 min if required, before being stimulated with 10 μg/ml goat polyclonal α-IgM F’ab fragment (Supplementary Table 8; Jackson Immunoresearch). Fluorescence emission (525 nm) was measured using a 488-nm laser and 530/30 filter on a BD LSRII flow cytometer.

B cell purification and sorting. B cells from spleen or peripheral lymph nodes were isolated with a B cell Isolation Kit from Miltenyi Biotec. To purify specific B cell subsets, cells were subsequently sorted using a BD FACSaria III or a BD FACSaria Fusion, using staining described above.

DNA isolation, RNA extraction and RT-qPCR assays. Total RNA was extracted from purified B cells using TRIzol (LifeTech) or RNeasy Micro or Mini Kit (Qiagen). RNA was treated with DNase I before reverse transcription into cDNA. ZFP36 family expression was analyzed using custom and commercially available TaqMan assays with specific primers (Supplementary Table 9). Expression of mRNA was calculated using a standard curve and was normalized to the expression of mRNA encoding β₂-microglobulin. Genomic DNA was extracted from purified B cells using Cell lysis solution (Qiagen) containing proteinase K (Roche). Protein was removed by salt precipitation, and the DNA was isolated using isopropanol. Relative abundance of Zfp36l1 exon 2 was analyzed by quantitative PCR with specific primers (Supplementary Table 9), and qPCR assays were performed with Platinum SYBR Green qPCR SuperMix (Life Technologies). Relative abundance of Zfp36l1 was calculated using the comparative threshold cycle (ΔACT) method and results were normalized to the expression of the control gene encoding TRBP.

Immunofluorescence. Spleens were frozen in OCT compound on dry ice and were sectioned on the cryostat (7–10 μm thick). Sections were air dried overnight, then fixed in ice-cold acetone for 15 min at 4°C. Tissue sections were rehydrated in PBS for 10 and were blocked in 5% NRS. Sections were stained in 5% NRS at 4°C overnight in a humidified chamber to detect IgM and MOMA-1, or CD1d, IgD and MOMA-1. Slides were washed PBS and were mounted in ProLong Gold antifade reagent (Thermo Fischer). Images were acquired at ×10 or ×20 magnification using a DeltaVision widefield fluorescence microscope (GE Healthcare). Images were quantified using Image-J software (NIH). MZ width was measured using Image-J software. At least ten follicles per genotype were imaged; measurements were taken from the edge of the MZ-1 cells to the edge of CD1d+ staining. Many measurements were taken per follicle to account for variability. The MFI of CD1d (minus background) was calculated using ImageJ (NIH). Follicular area was defined using staining of MOMA-1 (CD169). MFI was then calculated for this area (Supplementary Fig. 8e). MZ area was defined as the CD1d+ area outside staining of MOMA-1 and IgD (Supplementary Fig. 8e). Background fluorescence was calculated and subtracted from MFI values for MZ and FO.

Library preparation and high-throughput sequencing. Sorted MZ or Fo B cells from individual control mice were independently processed for RNA extraction. RNA-seq libraries were obtained using a TruSeq Stranded mRNA Sample Prep Kit (Illumina) or SMARTer Ultra Low Input RNA v4 and SMARTer Low Input Library Preparation Kit V2 (Clontech). Low-input libraries were prepared and sequenced from total RNA at Aros Applied Biotechnology A/S. RNA-seq libraries were sequenced using the HTSeq2000 (Illumina). 100–bp single-end or paired-end sequencing was performed on all libraries.

Bioinformatics for RNA-seq. Quality of sequencing data was analyzed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads were mapped to mouse genome (GRCm38) using TopHat2 (ref. 55). Reads aligning to genes were counted using htseq-count28, and analysis of differentially expressed genes was performed using the DESeq2 (Ribonouchard package)37. Reads were visualized using Integrative Genomics Viewer (IGV)56,59. Gene set enrichment analysis was performed using TopGene60 and the enriched GO biological processes were visualized using Revigo61. ICLIP data was analyzed as previously described25.

To analyze conservation of ZFP36L1-binding motif in its target genes, the 3′ UTR sequence of selected gene in mouse was queried against synthetic sequences in eutharian mammals using Ensembl Perl API.
Biological data analysis. ChIP-seq data for KLF2 (ref. 35) and IRF8 (ref. 33) and Affymetrix data for Klf2-deficient FO B cells14 were downloaded from the Gene Expression Omnibus. The Affymetrix data was analyzed using R/Bioconductor package affy62. Reads from ChIP-seq data were mapped to mouse genome (GRCm38) using Bowtie2 (ref. 63) and peaks were called using MACS2 (ref. 64). Only reproducible peaks were considered. Peaks were visualized using the Integrative Genomics Viewer.

Statistical analysis. Mann-Whitney tests were performed for statistical analysis of non-sequencing data. Additional details regarding sample size and statistics used have been provided in the figure legends where relevant.

Data availability. The RNA-seq data are available in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/gds) under the following accession codes: GSE79632 (Transcriptome of Zfp36l1-deficient MZ B cells, WT MZ B cells and WT FO B cells), and GSE79633 (Transcriptome of GFP-ZFP36L1 expressing and WT FO B cells). ChIP-seq data for KLF2 (ref. 35) and IRF8 (ref. 33) and Affymetrix data for Klf2-deficient FO B cells14 were downloaded from the Gene Expression Omnibus. The data that support the findings of this study are available from the corresponding author upon request.

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