The transcriptional network regulating antibody-secreting cell (ASC) differentiation has been extensively studied, but our current understanding is limited. The mechanisms of action of known “master” regulators are still unclear, while the participation of new factors is being revealed. Here, we identify Zbtb20, a Bcl6 homologue, as a novel regulator of late B cell development. Within the B cell lineage, Zbtb20 is specifically expressed in B1 and germinal center B cells and peaks in long-lived bone marrow (BM) ASCs. Unlike Bcl6, an inhibitor of ASC differentiation, ectopic Zbtb20 expression in primary B cells facilitates terminal B cell differentiation to ASCs. In plasma cell lines, Zbtb20 induces cell survival and blocks cell cycle progression. Immunized Zbtb20−/− deficient mice exhibit curtailed humoral responses and accelerated loss of antigen-specific plasma cells, specifically from the BM pool. Strikingly, Zbtb20 induction does not require Blimp1 but depends directly on Irf4, acting at a newly revealed. Here, we identify Zbtb20, a Bcl6 homologue, as a novel regulator of late B cell differentiation and provide new insights into this complex process.

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necessary and sufficient for B cells to differentiate fully into ASCs (Turner et al., 1994; Shaffer et al., 2002; Shapiro- Shelef et al., 2003). Once induced, Blimp1 represses Pax5, Bcl6, and Bach2, extinguishing the B cell phenotype and enforcing ASC differentiation. Blimp1 blocks proliferation through repressing c-Myc (Lin et al., 1997) and indirectly induces Xbp-1, a factor critical for the unfolded protein response that enables high-level antibody secretion (Shaffer et al., 2004; Taubenheim et al., 2012).

Irf4 expression is maintained at a low level by Mitf in mature B cells (Lin et al., 2004) and is further down-regulated in GC B cells. Nevertheless, Irf4 is required for the generation of GCs and for CSR (De Silva et al., 2012; Ochiai et al., 2013), and is essential for plasma cell development (Klein et al., 2006; Sciammas et al., 2006).

Despite these advances, it is still unclear how Irf4 controls the transition from B cell to ASCs. Irf4 is not essential for CSR, but is critical to induce high-level antibody secretion (Shaffer et al., 2001; Taubenheim et al., 2012). Furthermore, as immature pre-plasmablasts can arise from Blimp1-deficient B cells, Blimp1 cannot be the factor that initiates the program of ASC differentiation, though it is required for its completion (Kallies et al., 2007). Conversely, induction of Blimp1 in the absence of Irf4 fails to drive plasma cell differentiation (Klein et al., 2006). Thus, the current model of the transition from B cell to ASCs is incomplete, with other factors likely to be involved (Klein and Dalla-Favera, 2007).

The present study identifies Zbtb20 as a new regulator of plasma cell differentiation. This protein, also named Zip288, DPZF (Zhang et al., 2001), and HOF (Mitchelmore et al., 2002), is a broad complex, Tramtrack, Bric-à-brac, and Zinc Finger (BTB-ZF) protein, homologous to Bcl6. BTB-ZF proteins are an emerging group of regulators, acting mainly as repressors, in many aspects of development, cancer, and lymphoid lineage differentiation (Kelly and Daniel, 2006; Costoya, 2007; Beaulieu and Sant’Angelo, 2011).

Zbtb20 was originally identified in human dendritic cells (Zhang et al., 2001) and in the developing central nervous system (Mitchelmore et al., 2002). Two different isoforms, Zbtb20S and Zbtb20L, are generated by alternative splicing and translational start sites (Mitchelmore et al., 2002), but are indistinguishable in terms of function (Nielsen et al., 2007). They can dimerize in vitro via their BTB domains, are nuclear and bind to DNA via the five Krüppel-type zinc finger domains (Mitchelmore et al., 2002). Zbtb20-deficient mice exhibit peripheral lethality, with early growth abnormalities (Sutherland et al., 2009; Rosenthal et al., 2012). Conditional deletion of Zbtb20 in the liver highlights its involvement in metabolism (Xie et al., 2008; Peterson et al., 2011), and in the nervous system, Zbtb20 is involved in cortex formation and neuronal specification (Nielsen et al., 2007; Rosenthal et al., 2012). To date however, no role for Zbtb20 in the adaptive immune system has been described.

Here, we show that within the B cell lineage, Zbtb20 is constitutively expressed in peritoneal B1 cells, but not expressed in follicular B cells. It is induced in GC B cells and is highest in mature plasma cells of the BM. Ectopic expression of Zbtb20 in cell lines and in primary B cells promotes the plasma cell differentiation program and impacts critical aspects of late B cell differentiation, such as cell proliferation and survival. Whereas B1 and GC cells develop normally in Zbtb20-deficient mice, Zbtb20 loss curtails antibody responses upon immunization and causes a selective disappearance of BM ASCs over time. Interestingly, Zbtb20 expression is dependent on Irf4, which binds to a newly identified Zbtb20 promoter specifically active in ASCs.

RESULTS
Identification of a new BTB-ZF gene highly expressed in plasma cells
A deep understanding of ASC biology is hampered by their rarity and by the paucity of markers available to purify them. To identify new plasma cell–specific molecules, resting B cells, and ASCs at different stages of differentiation were isolated from the spleen and BM of Blimp1/GFP reporter mice (Kallies et al., 2004). Microarray analyses revealed that, like genes known to be induced in ASCs, such as Sdc1 (syndecan-1), Xbp1, and Irf4, the transcriptional repressor Zbtb20 was strongly up-regulated during plasma cell differentiation (Fig. 1 A).

Graded expression of Zbtb20 in follicular B cell response
We performed quantitative PCR (qPCR) for Zbtb20 on sorted cells representing different stages of B cell development. Zbtb20 mRNA was low in developing B cells, but highly expressed in peritoneal B1 cells, GC B cells, and in splenic and BM plasma cells (Fig. 1 B). Using our in-house generated monoclonal antibody in intracellular flow cytometric analysis, we confirmed that all populations expressing Zbtb20 mRNA were positive at the protein level (Fig. 1 C). Zbtb20 expression progressively increased from B1 and GC cells, through immature Blimp1/GFP plasmablasts in the spleen to Blimp1/GFP plasma cells in the BM (Fig. 1 D). Consistent with those data, immunohistochemistry on spleen after NP–KLH immunization revealed that weak Zbtb20 staining colocalized with GC structures, whereas strong staining could be observed in individual cells (Fig. 1 E). Co-staining with an anti–syndecan-1 antibody revealed that some of these cells were also syndecan-1–, and hence likely ASCs (unpublished data). These data identify Zbtb20 as a transcription factor constitutively expressed in B1 cells and induced upon antigen dependent activation of follicular B cells, culminating with highest expression in mature ASCs.

Zbtb20 expression in cell lines reflects its primary B cell profile
As the B lineage cells that normally express Zbtb20 in vivo are rare or difficult to culture, we used cell lines representing different stages of B cell differentiation as tools to discover potential functions of Zbtb20 within the B cell lineage. Zbtb20 expression was low in the immature WEHI231 and the mature I29B cell lines, but increased in A20, a GC B cell lymphoma (with high Bcl6 and AID, low Blimp1, and Irf4 expression; Fig. 2 A). Consistent with our ex vivo data, most plasmacytomas
plasma cell line, which expresses little endogenous Zbtb20. Two clones were generated for each construct. Ectopic expression of Zbtb20 and translocation of Zbtb20 to the nucleus upon estradiol treatment were confirmed (Fig. 2 C and not depicted).

Microarray screens were performed with the P3 cell lines 6 and 48 h after estradiol treatment to identify Zbtb20 regulated genes. Ingenuity Pathway Analysis revealed that the cell cycle and life span pathways were most affected by increased Zbtb20 expression in P3 cells (unpublished data). Genes controlling proliferation, such as cyclin-dependent kinases (CDKs) p15, p16, p21, and cell survival genes including Bcl-w and Pim2, were most affected among the array probes (Fig. 3 A). Quantitative PCR analyses suggested that the main CDK regulated by Zbtb20 is p15 (Fig. 3 B). We performed in vitro proliferation assays to determine whether the modulation of cell cycle regulators by Zbtb20 had a biological impact on the cells. The different clones of P3 cells were cultured (±estradiol) and the cells counted daily. Strikingly, when Zbtb20 was induced in P3/Zbtb20-ER cells, their numbers decreased significantly.

Zbtb20 affects cell survival and cell proliferation in a plasma cell line

A retroviral vector was designed to overexpress Zbtb20 in an inducible manner (Fig. 2 B). Sequences encoding Zbtb20 and the human estradiol receptor (ER) dimerization domain were fused, enabling inducible nuclear translocation and activation upon estradiol treatment. An analogous vector expressing Zbtb20, minus its DNA-binding Zinc Finger domain (ΔZF), fused to ER, was used to assess the dependence of Zbtb20 function on direct DNA binding. We introduced these vectors into the P3 plasma cell line, which expresses little endogenous Zbtb20. Two clones were generated for each construct. Ectopic expression of Zbtb20 and translocation of Zbtb20 to the nucleus upon estradiol treatment were confirmed (Fig. 2 C and not depicted).

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(J558, MPC11, and ST33MM) displayed high Irf4 and Blimp1 expression, and expressed Zbtb20 in the nucleus. Only plasmacytoma P3 exhibited low Zbtb20 levels. All the cells that were positive for Zbtb20 expressed both isoforms equally (Fig. 2 A and not depicted). This strong correlation between Zbtb20 expression in primary B cells and their cell line counterparts suggested that the latter were appropriate models to explore Zbtb20 function during B cell differentiation.
over time compared with controls. DNA binding was required for the response (Fig. 3 C). We performed cell cycle analyses and found that reduced proliferation in P3/Zbtb20-ER cultures correlated with an increased percentage of cells in the G1 phase of the cycle. Kinetic experiments revealed that upon introduction to culture, ~50% of untreated P3/Zbtb20-ER cells quickly left G1 to enter the G2, M, and S phases, whereas Zbtb20 induction detained cells in G1 (Fig. 3 D).
Quantitative PCR was also performed to confirm the Zbtb20-mediated effect on expression of survival genes identified by microarray. Bcl-u, Mel-1, Bel-x, Pin2, Bama (Tfifsi17), and Bcl2 were indeed induced by Zbtb20 in the plasma cell line (Fig. 4 A), and in all cases required the Zbtb20 DNA-binding domain (Fig. 4 A). To determine whether Zbtb20 induction confers resistance to stress-induced cell death, the P3 clones were cultured for 48 h (±estradiol) and subsequently exposed to the DNA damage-inducing agents etoposide or adriamycin for 24 h. Both of these drugs caused a strong increase in cell death accompanied by caspase 3 activation in the control cell lines (Fig. 4, B and C). Upon Zbtb20 induction, the percentages of both PI- and active caspase 3+ cells were significantly reduced, showing that Zbtb20 can promote cell survival (Fig. 4, B and C). To determine whether the modulation of survival mediated by Zbtb20 is restricted to P3 cells, or can be generalized to other cell types, we overexpressed Zbtb20 in the immature WEHI231 B cell lymphoma and the plasmacytoma MPC11. The modulation of plasma cell genes was assessed by qPCR. Inducing Zbtb20 in WEHI231 did not affect cell survival or cell cycle gene expression. However, in the plasmacytoma cell line MPC11, the induction of Zbtb20 led to similar effects as in the P3 cell line (Fig. 4 D and not depicted). These results suggest that Zbtb20 is a potential cell stage–specific modulator of cell cycle and cell survival through the regulation of key genes involved in these critical processes.

Zbtb20 promotes plasma cell differentiation in primary B cells

To ask whether Zbtb20 could influence gene expression and differentiation in primary B cells, we generated a constitutively active Zbtb20 retroviral vector coexpressing mCherry and infected LPS activated splenic B cells from Blimp1gfp/+ reporter mice. Zbtb20 overexpression strongly accelerated the differentiation of B cells into Blimp1/GFP+ plasma cells, which were also syndecan-1+ and MHCII+ and secreted Ig (Fig. 5, A–C). mCherry+ cells harvested from LPS cultures 3 d after infection with control or Zbtb20 retroviruses were sorted and the expression of several genes was measured. As expected, the
Not induce or increase the antibody secretion in either mutant (Fig. 5, E and F; and not depicted). Thus, while Zbtb20 facilitates Blimp1 induction and initiation of differentiation, it is not sufficient to trigger full plasma cell differentiation in the absence of Blimp1 or Irf4.

Zbtb20 is required for a normal humoral immune response

To assess the function that Zbtb20 plays in the B cell compartment in vivo, and to circumvent the fact that Zbtb20 KO mice die perinatally, we reconstituted sublethally irradiated Rag1−/− or Rag2−/−/γc−/− mice with fetal liver cells from Zbtb20 KO (Rosenthal et al., 2012) or WT littermate embryos. Analysis of the B cell compartment revealed normal cell populations in the spleen and BM of mice from both groups (unpublished data). B1 cells in the peritoneal cavity, which normally express Zbtb20, were found at normal levels in the mutants (Fig. 6 A). However, using the Blimp1/GFP reporter, we found that the elevated Blimp1 levels that are characteristic of normal B1 cells (Fairfax et al., 2007) were not maintained.

Figure 5. Zbtb20 promotes plasma cell differentiation in primary B cells. (A) Purified B220+ Blimp1gfp/+ cells were cultured for 24 h with LPS and transduced with a control or Zbtb20-encoding vector expressing mCherry as a reporter. The percentage of Blimp1/GFP expressing cells among the mCherry+ cells was assessed at the indicated times. Circles represent mean ± SD from three independent experiments. (B) 48 h after transduction, mCherry+ cells were analyzed for the expression of Blimp1/GFP, syndecan-1, and MHCII. The percentage of cells in each gate is indicated. Data are representative of six independent experiments. (C) mCherry+ cells were sorted on day 2 after retrotransduction and analyzed by ELISpot for the frequency of IgM-secreting cells. Bars represent mean ± SD of three independent experiments. (D) mCherry+ cells were sorted on day 2 after retrotransduction and assessed by qPCR for expression of Blimp1/GFP, syndecan-1, and MHCII. The percentage of cells in each gate is indicated. Data are representative of three independent experiments. (E) Blimp1-deficient B220+ cells were transduced with a control or Zbtb20-encoding vector. After 3 d, transduced cells were analyzed for the expression of Blimp1/GFP and syndecan-1 expression among mCherry+ cells. The percentage of cells in each gate is indicated. Data are representative of three independent experiments. (F) Irf4−/− Blimp1/GFP reporter B220+ cells were transduced with a control or Zbtb20-encoding vector. After 3 d, transduced cells were analyzed for Blimp1/GFP and syndecan-1 expression among mCherry+ cells. Data are representative of three independent experiments.
in Zbtb20-deficient cells (Fig. 6 A), and that, when cultured in vitro with LPS or CpG, Zbtb20-deficient B1 cells differentiated less efficiently into syndecan-1+ ASCs than WT B1 cells (Fig. 6 B). Total serum immunoglobulin levels in naive mice, measured by Igk ELISA, were reduced by 4–5-fold in Zbtb20-deficient mice (18.4 ± 2.8 mg/ml for WT compared with 3.9 ± 0.4 mg/ml for mutants; \( n = 4 \) for each), with reductions across all isotypes tested (Fig. 6 C and not depicted). ASC numbers in naive mice, estimated by ELISpot, were also significantly reduced (Fig. 6 C).

We immunized reconstituted WT and Zbtb20 KO mice with NP-KLH to determine whether Zbtb20 KO mice were

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Figure 6. Defective B cell response in Zbtb20 KO mice. (A) Flow cytometric analyses of peritoneal cavity (PerC) cells to determine the percentage of B1 (B220\(^{hi}\), CD23\(^{-}\)) and B2 (B220\(^{+}\) CD23\(^{+}\)) cells from WT and Zbtb20 KO mice expressing the Blimp1 GFP reporter. Intracellular staining shows the level of Zbtb20 in each of these populations, and Blimp1 levels are shown using the GFP reporter. Results are representative of at least two independent experiments using three mice of each genotype. (B) B1 cells from WT and Zbtb20 KO mice were cultured with CpG or LPS for 2 d. The percentage of syndecan-1+ cells was assessed by flow cytometry. Bars represent the mean ± SD from seven mice analyzed in three independent experiments. **, \( P = 0.0065 \); **, \( P = 0.0052 \). (C) Total serum Ig (measured as Igk) in naive mice, and ASCs as determined by Igk ELISpot. Bars represent the mean ± SD from three independent experiments, each using two mice. **, \( P = 0.0043 \); * , \( P = 0.0329 \); ** , \( P = 0.0257 \). (D) Analysis of spleen cells 7 d after NP-KLH immunization in WT and Zbtb20 KO mice. Cells were gated as B220\(^{+}\) lymphocytes or as Blimp1\(/\)GFP\(^{+}\) plasmablasts, and the level of NP staining was assessed on the gated cells. Data are representative of five to six mice per genotype analyzed in two independent experiments. (E) Titers of NP-specific IgG1 in serum of immunized WT (\( n = 4 \)) or KO (\( n = 5 \)) mice over \( > 4 \) mo. Bars represent mean ± SD. Representative of two independent experiments. (F) NP-specific IgG1 ASCs identified by ELISpot assay in spleens and BM of mice at the times indicated. Bars represent mean ± SD (\( n = 3 \) to 5 mice for each group). Representative of two independent experiments. * , \( P = 0.03 \), calculated using the Mann-Whitney \( U \) test. (G) Sorted ASCs were stained for BrdU incorporation. Bars represent mean ± SD from three mice per genotype and data are representative of two independent experiments. (H) Cell cycle analysis of sorted ASCs. Bars represent mean ± SD from three mice per genotype and data are representative of two independent experiments. (I) qPCR on sorted plasma cell populations for the indicated genes. Bars represent mean ± SD from three mice per genotype and data are representative of two independent experiments.
able to generate normal humoral immune responses. GCs developed similarly in the presence or absence of Zbtb20 (5.6 ± 1.2% of B220+ cells for WT compared with 5.3 ± 1.2% for mutants at day 7) and were maintained at similar level up to 21 d after immunization (unpublished data). The percentages of centroblasts and centrocytes in the GCs, and of apoptotic GC B cells in spleen, were not affected by Zbtb20 loss (unpublished data). NP-specific B cells (B220+/BlimpGFP+) and plasmablasts (Blimp1/GFP+) were detected at day 7 in similar numbers in mice of both genotypes, indicating that the initial response to immunization was not impacted by Zbtb20 loss (Fig. 6 D). At this early time point, the GC response is only just beginning, so these data imply that the extrafollicular ASC response is intact in the mutants. Initially, serum anti-NP IgG1 titers were also similar between KO and WT mice (Fig. 6 E), and the frequencies of ASCs secreting high-affinity anti-NP IgG1 antibody were comparable (unpublished data), indicating normal affinity maturation in Zbtb20-null mice. Strikingly, however, the KO mice were unable to achieve the same anti-NP antibody titers as controls at the height of the response (peaking at ~5-fold lower levels than controls). Furthermore, whereas WT mice retained high anti-NP IgG1 titers for the duration of the experiment, titers in KO mice declined markedly from the lower peak, and finished more than eightfold lower than in WT mice (Fig. 6 E). Interestingly, although the frequencies of NP-specific ASCs in spleen and BM were equivalent on day 14 in WT and KO mice, at later time points (days 118 [not depicted] and 156 [Fig. 6 F]), the KO mice had significantly (>30-fold) fewer ASCs in BM than controls. ASC numbers in the spleens of KO mice were also consistently reduced (to ~60% of WT levels), but the difference did not reach statistical significance (Fig. 6 F). These in vivo results indicate that, under this immunization regimen, that Zbtb20 is not essential for an initial GC, plasma cell, and antibody response. However, in its absence, the early antibody response is blunted and, later, ASC loss in the BM is significantly and specifically accelerated, causing serum Ig levels to wane more rapidly.

We performed several experiments to further characterize the difference between ASCs in WT and KO mice. Antigen-specific ASCs lose antigen receptor expression upon differentiation, as observed using the Blimp1/GFP reporter gene. It is therefore not possible to distinguish recently generated from long-lived ASCs based on surface staining. We therefore measured ASC generation in vivo using BrdU labeling. We did observe the described differences (Kallies et al., 2004) in BrdU incorporation into the cycling splenic Blimp1/GFP+ plasmablasts and the noncycling Blimp1/GFPhi plasma cells of spleen and BM. We did not see significant differences in the percentage of BrdU+ cells in these three populations comparing WT and KO mice, although the KO mice consistently exhibited higher BrdU incorporation in the BM ASC compartment (Fig. 6 G). This is likely to indicate a modest elevation in the influx of recently generate ASCs into the BM, as cell cycle analyses of BM ASCs gave identical results for WT and Zbtb20−/− cells (Fig. 6 H).

We sorted total syndecan1+/Blimp1/GFP+ plasmablasts and plasma cells from spleen and BM of WT and KO mice and performed qPCR for genes known to be important for ASC longevity, such as Bma and Mel1 (O’Connor et al., 2004; Peiperzak et al., 2013). These were not reduced in Zbtb20 GC B cells or spleen and BM ASC compared with controls (Fig. 6 I). KO GCs and ASCs also expressed normal levels of Bcl2, Bcl2l1/BclX, and Bcl2l11/Bim compared with their WT counterparts (unpublished data). Together, and with the caveat that we have compared total ASCs from WT and KO mice without knowledge of their ages, these data suggest that Zbtb20 does not strongly affect the generation of ASCs, their capacity to enter a quiescent state, or the expression of known mediators of ASC survival. Zbtb20 is, however, essential for the maintenance of a long-lived antigen-specific BM pool of ASCs and of persistent antigen-specific Ig in serum.

Zbtb20 expression is dependent on Irf4 but independent of Blimp1 To find where Zbtb20 lies in the genetic hierarchy of ASC differentiation, Blimp1−/− B cells were activated with LPS and Blimp1/GFP-positive and -negative cells were sorted. Both qPCR and FACS analyses consistently showed a slight increase in Zbtb20 expression in GFP+ Blimp1−/− control plasmablasts (Fig. 7 A, left; and not depicted). In activated Blimp1-deficient B cells, Zbtb20 expression was higher in the GFP+ population than in the equivalent control cells (Fig. 7 A, compare middle and left). Blimp1 is therefore not required for Zbtb20 expression; indeed, its loss increases Zbtb20 expression. As Zbtb20 up-regulates Blimp1 (Fig. 5 A), this implies a complex regulatory relationship between the two repressors. In striking contrast, Irf4 deficiency led to an almost complete loss of Zbtb20 expression in vitro, both in Blimp1/GFP− and Blimp1/GFP+ cells (Fig. 7 A).

We then asked whether Zbtb20 expression required Blimp1 or Irf4 in vivo. Because ASCs do not form in the absence of Irf4 or Blimp1, and because GCs require Irf4, we focused on peritoneal B1 cells as a B cell population that expresses Zbtb20 in vivo. B1 cells were evident in mice of each genotype (Fig. 7 B). However, while control and Blimp1-deficient B1 cells expressed Irf4 or Blimp1, and because GCs require Irf4, we focused on peritoneal B1 cells as a B cell population that expresses Zbtb20 in vivo. B1 cells were evident in mice of each genotype (Fig. 7 B). However, while control and Blimp1-deficient B1 cells expressed Zbtb20, Irf4-deficient B1 cells did not, confirming the Irf4 dependence of Zbtb20 expression in vivo.

We ectopically expressed Irf4 in the mature I29B cell line, which expresses little Irf4 or Blimp1, and because GCs require Irf4, we focused on peritoneal B1 cells as a B cell population that expresses Zbtb20 in vivo. B1 cells were evident in mice of each genotype (Fig. 7 B). However, while control and Blimp1-deficient B1 cells expressed Zbtb20, Irf4-deficient B1 cells did not, confirming the Irf4 dependence of Zbtb20 expression in vivo.

Irf4 directly binds to a Zbtb20 promoter Zbtb20 is expressed from a complex gene spanning >750 kbp. RNA sequencing on brain tissue and two cell lines showed that while transcription starts far upstream in the locus in brain,
Computational analyses (TransFacPro) identified putative Irf4 binding sites near the B1/ASCs Zbtb20 transcriptional start site and elsewhere in the locus (Fig. 7 E). Chromatin immunoprecipitation from two plasmacytomas (MPC11 and J558) with anti-Irf4 antibody showed a strong enrichment of sequences preceding the B1/ASCs transcriptional start site, but not of other putative Irf4 binding sites (Fig. 7 F).

in the GC-like A20 lymphoma and MPC11 plasmacytoma, transcripts were restricted to downstream regions of the locus (boxed in Fig. 7 D). 5′ RACE on A20 and MPC11 identified two major and distinct start sites from as yet unmapped exons (Fig. 7 E). RNA-Seq analysis on ex vivo purified B1, GCs and plasma cells showed profiles of Zbtb20 expression matching their cell line counterparts, with peaks in these new exons (Fig. 7 E). Thus, Zbtb20 is differentially regulated in GCs and B1/ASCs.

Figure 7. Irf4 binds to the promoter of Zbtb20 to trigger its expression. (A) B cells from the indicated mice were cultured for 3 d with LPS, sorted according to Blimp1/GFP expression and stained for intracellular Zbtb20 levels. Data are representative of three independent experiments on two mice of each genotype. (B) Analysis of peritoneal cells of the indicated mice to identify B1 and B2 cells. Histograms show Zbtb20 levels for both populations. Representative of three independent experiments, with three mice for each genotype. (C) I29B cells were transduced with an Irf4/GFP or a control vector and sorted at day 3 according to GFP level. Zbtb20 levels were assessed by FACS. Representative of three independent experiments. (D) RNA-Seq analysis of brain tissue, A20, and MPC11. Structure of the Zbtb20 gene according to the Ensembl database is depicted. The presence of gene NR_002445 is indicated (gray box). Arrows shows alternative Zbtb20 translational start sites (TSS). Representative of one experiment. (E) Identification of Zbtb20 transcriptional start sites in MPC11 and A20 cells by 5′RACE PCR. 18 independent clones from A20 and 45 clones from MPC11 cell lines were bidirectionally sequenced. Their location and frequency among the sequenced clones are shown. RNA-Seq was performed on the indicated cell lines and primary B cells. Putative transcriptional start sites in GC (blue dotted box) and ASC/B1 (green dotted box) are indicated. Locations of sequences (#1–#5; arrowheads) containing putative Irf4 binding sites are depicted. (F) Anti-Irf4 ChIP was performed on the indicated cell lines and assessed by qPCR using primers 1–5 listed in Table S5. Data are representative of two independent experiments.
differentially in GC B cells and ASCs, with Irf4 directly activating the gene from a newly identified plasma cell-specific promoter.

DISCUSSION

Terminal B cell differentiation is a complex process currently modeled upon the actions of a small number of "master regulators," and the gaps in our understanding are clear. Insight into the mechanism of differentiation, both its initiation and its full execution, is advanced with the identification of each new contributing factor.

Here, we identify Zbtb20 as a new mediator of B cell differentiation specifically expressed in B1 and GC B cells and ASCs. Zbtb20 is a BTB-ZF transcription factor, and other members of the family have been shown to be active within the B cell lineage (Chevrier and Corcoran, 2014). For instance, early B lineage commitment is mediated by LRF, Bcl6, and Miz-1 (Maeda et al., 2007; Duy et al., 2010; Kosan et al., 2010), MZ B cell differentiation is controlled by LRF (Sakurai et al., 2011), and the GC reaction is driven by Bcl6 and LRF (Fukuda et al., 1997; Sakurai et al., 2011). Finally, Zbtb32 has been associated with plasma cell differentiation (Yoon et al., 2012).

In activated conventional B cells, which express little Zbtb20, its enforced expression increased Blimp1 induction and strongly promoted plasma cell differentiation. In contrast, Zbtb20 is constitutively expressed in B1 cells. These sentinel cells of the peritoneal and pleural cavities rapidly differentiate to ASCs upon antigen encounter. They are transcriptionally poised toward differentiation, with elevated Blimp1 and depressed Pax5 and Bcl6 levels compared with conventional B cells (Fairfax et al., 2007). We show that Zbtb20 is highly expressed in B1 cells, where it facilitates Blimp1 expression in vivo and efficient ASC differentiation in vitro. Interestingly, Zbtb32, which has recently been shown to influence plasma cell differentiation (Yoon et al., 2012), is also highly expressed in B1 cells (unpublished data). These data suggest that Zbtb20 and Zbtb32 may contribute to the special characteristics of B1 cells. Interestingly, MZ B cells, which are also able to rapidly differentiate into ASCs in vitro, express low levels of both Zbtb20 and Zbtb32 (Fig. 1 A and not depicted). Therefore, in this B cell subpopulation, rapid activation and differentiation is likely to depend on a different transcriptional pathway. For example, MZ B cells do not down-modulate Pax5 or Bcl6 levels as strongly as B1 cells do (Fairfax et al., 2007; and unpublished data). The difference in cell behavior is also consistent with the fact that B1 and MZ B cells have different developmental precursors and differential requirements for key transcription factors, such as Oct2, Obf-1, and LRF, for their generation (Humbert and Corcoran, 1997; Samardzic et al., 2002; Sakurai et al., 2011).

The effects of Zbtb20 on plasma cell differentiation, cell cycle arrest, and cell survival are opposite to the role played by Bcl6. BTB-ZF transcription factors are known to be able to heterodimerize through their BTB domains (Costoya, 2007) and part of the function played by Bcl6 in GC depends on its interaction with the BTB-ZF transcription factor Miz1 (Phan et al., 2005; Saito et al., 2009). Due to its high expression in GC, Zbtb20 would be an obvious candidate to counteract Bcl6 function and promote GC exit. However, our in vivo data using NP-KLH immunization did not identify a role for Zbtb20 in the generation of the GC, in the switch between dark zone and light zone, or in the frequency of apoptotic cells. Alternatively, Zbtb20 may oppose the repressive influence of Bcl6 on Blimp1 expression and initiation of ASC differentiation. Because we have not observed physical interactions between Zbtb20 and Bcl6 or Miz1 (unpublished data), this is unlikely to be via direct binding between Zbtb20 and Bcl6, but may reflect competition for binding to common gene targets. Thus far, the function Zbtb20 plays in GC remains elusive and requires further investigation.

Zbtb20 is expressed at its highest level in mature, non-dividing, long-lived plasma cells in vivo. In transformed plasma cells, Zbtb20 increased expression of genes known to be critical for ASC survival in vivo, such as Bona and Mll1 (O'Connor et al., 2004; Peperzak et al., 2013), and promoted survival to cytotoxic agents. It also induced expression of the CDK inhibitor p15, leading to cell cycle blockade. Interestingly, other reports have linked BTB-ZF proteins to the regulation of CDK inhibitors (Phan et al., 2005; Weber et al., 2008; Jeon et al., 2009). Collectively, our in vitro data suggest that Zbtb20 may act to repress mitosis and promote the survival of plasma cells. In vivo, loss of Zbtb20 did not dramatically affect induction of antigen-specific plasma cells, isotype switching, affinity maturation, or the migration of plasma cells to the BM. However, the persistence of antigen-specific plasma cells, as measured by their output in the serum and their maintenance in the BM (and to a lesser extent in the spleen), was significantly reduced in the absence of Zbtb20.

In primary ASCs taken ex vivo, expression of Bona and Mll1, two potent mediators of plasma cell survival (O'Connor et al., 2004; Peperzak et al., 2013) was not affected by the absence of Zbtb20. Similarly, BrdU labeling and cell cycle analysis did not reveal a dramatic effect of Zbtb20 on ASC cell cycle parameters. There was a suggestion that the BM pool of Zbtb20â/â ASCs contained more newly generated cells, which could account for the observed depletion of antigen-specific ASCs in the Zbtb20â/â BM pool. Manz et al. (2005) describe competition for limited survival niches in the BM between existing and newly generated plasma cells. If the number of new ASCs entering the BM is elevated in Zbtb20 deficiency, possibly as a result of its role in modulating cell cycle or cell survival genes in the short term, the newcomers may replace the antigen-specific ASC induced by immunization more rapidly than in normal mice. The observation would have to be confirmed through the tracking of ASCs from their generation until their death, which is currently technically challenging. To fully understand how Zbtb20 affects ASC maintenance, it will be necessary to identify its direct targets in plasma cells. In addition, because our in vitro studies indicated that the effect of Zbtb20 on cell survival is context dependent, it is likely that this effect requires as yet unidentified cofactors of Zbtb20, expressed in ASCs but not B cells.
A recent study showed that Pax5 binds in the Zbtb20 promoter, which suggests that Pax5 could contribute to Zbtb20 silencing in immature B cell type (Revilla-I-Domingo et al., 2012). This concurs with our observation that Zbtb20 is not expressed during B cell development. However, because the presence of Pax5 in GCs, B1, and early plasma cells does not prevent Zbtb20 expression, Pax5 is not sufficient to repress Zbtb20. We show here that Zbtb20 induction is dependent on Irf4 in B1 cells and during plasma cell differentiation. We mapped two new transcriptional start sites, one specifically active in plasma cells and B1 cells and the other in GC B cells, in newly described exons. B1 and ASCs share a Zbtb20 promoter that contains a Pu-1/Irf4 consensus site to which Irf4 binds in vivo. We propose that direct binding of Irf4 to this site drives Zbtb20 expression in B1 and ASCs. Given the absence of Irf4 in GC B cells, induction of Zbtb20 in GCs likely acts through other factors.

The relationship between Blimp1 and Zbtb20 expression is more perplexing. We show that Zbtb20 overexpression increases the number of Blimp1-expressing B1 cells in vivo and in culture. We also show that, in the absence of Blimp1, Zbtb20 is expressed normally in B1 cells, but in vitro activated cells, its expression increases. This implies that Zbtb20 is upstream of Blimp1 and favors its expression, but that Blimp1 may be part of a negative feedback loop that regulates Zbtb20. Consistent with this, ChIP sequencing analyses showed that Blimp1 associates directly with the Zbtb20 locus both in mouse (unpublished data; M. Minnich and M. Busslinger, personal communication) and human, via a highly conserved domain (unpublished data; R. Tooze, personal communication). Although the expression of Zbtb20 in human plasma cells is not clear yet, this observation suggests that a similar pattern is shared between mice and human. Since Zbtb20 is expressed in certain human B cell lymphomas, it will be of interest to further investigate the exact expression pattern of Zbtb20 in the human B cell lineage.

In summary, our study has identified Zbtb20 as a new regulator of plasma cell differentiation. It is expressed in ASCs and their direct precursors and is Irf4 dependent. Zbtb20 has the capacity to impact several phenotypic properties of activated B cells, plasmablasts and mature plasma cells, including enhancing differentiation, slowing cell division, and bestowing ASC longevity via enhanced survival. Therefore, our data provide new insights into plasma cell biology and open the way to a more detailed analysis of the roles Zbtb20 plays in the fine-tuning of plasma cell differentiation and the maintenance of long-term humoral immunity.
Flow cytometry and antibodies. Single-cell suspensions were stained with the antibodies listed in Table S3 in the presence of 2.4G2 mAb to block Fc receptors. Propidium iodide (PI) or Sytox blue (Invitrogen) was used to exclude dead cells. Intracellular staining for Zbhb20 was performed using the Foxp3 Fixation/Permeabilization kit (eBioscience). FACS data were collected using a FACSCanto, LSR II, or Fortress (all from BD) and analyzed on FlowJo (Tree Star). For DNA content analysis, cells were fixed with the eBioscience kit, treated with RNase A for 45 min at 37°C, and stained with PI before FACS analysis. Cell sorting was performed on a MoFlo (Dako), FACS Vantage (BD), or FACS Arta (BD) and cell purity was typically >98% upon reanalysis.

Immunohistological analysis. For histological analyses, spleen samples were frozen in OCT (Tissue-Tek; Sakura) and 7-µm sections were stained with primary antibodies. The substrates used were as follows: AEC (Vector Laboratories), Fast Red (Biotech). The substrates used were as follows: AEC (Vector Laboratories), Fast Blue (Vector Laboratories), and DAB + chromogen (Dako).

BrdU labeling and cell cycle analysis. In vivo BrdU labeling and analysis was performed as described previously, except that mice were fed BrdU water for five rather than four days (Kallies et al., 2004). Cell cycle analysis was performed as described in Zotos et al. (2010).

ELISpot and Elisa. For ELISpot, 500 mCherry+ cells were purified by FACS 2 d after infection and added to a 96-well累累licate plate (Millipore) coated with 2 µg/ml DA (sheep anti–mouse Ig; Silenus Laboratories) and incubated for 4–5 h at 37°C and 10% CO2. IgM-secreting cells were identified with anti-IgM (biotin) and streptavidin-AP (Invitrogen). Spots were visualized with BCIP (Sigma-Aldrich) and counted using an automated reader (AID ELISpot Reader System, software version 4). After NP-KLH immunization, spleen and BM total cells were incubated for 5 h on plates previously coated with 20 µg/ml of NP13-BSA. Anti-NP IgG1 was detected using goat anti–mouse IgG1 conjugated to HRP (SouthernBiotech) and visualized as described above. ELISA was performed as described previously (Kallies et al., 2007).

RNA sequencing. RNA was extracted from mouse brain, A20, and MPC11 cell lines and ex vivo from peritoneal B220+CD23- B1 cells, follicular B cells (B220+CD23-) from spleen, BM Blimp-GFP/+syndecan-1- ASC and splenic PNA+ /FAS-/B220- GC B cells as above. Sequencing (100 bp, paired end) was performed at the Australian Genome Resource Centre, using a HiSeq2000 (Illumina). Reads were mapped to the mouse reference genome mm9 using the Subread aligner (Liao et al., 2013). Ten 16-bp subreads were extracted from each read and mapped without permitting mismatches. A successful hit was reported if three or more subreads from a read mapped to same genomic location. Exon–exon junctions were identified when different subreads from the same read mapped to different exons. Paired-end information was used in the mapping. On average, 94% of reads were successfully mapped. Table S4 gives the percentage of mapped reads for each library. Read depth plots were generated using Integrated Genome Browser (Nicol et al., 2009).

5′ RACE PCR. The transcriptional start site for the mouse Zbhb20 gene was mapped using a RACE cDNA amplification kit (Ambion). Gene-specific nested primers were chosen from a 5′ region of the coding region that is common to both isoforms. PCR products generated from the 5′ RACE PCR reaction were purified from an agarose gel and cloned. 18 independent clones from A20 and 45 clones from MPC11 cell lines were bidirectionally sequenced to ensure that microheterogeneities in sequence length were not overlooked.

CHIP PCR. Chromatin immunoprecipitation was performed as previously described (Emslie et al., 2008). In brief, 2 x 10^6 A20, MPC11 and J558 cells were fixed in 1% formaldehyde and quenched with 10% of glycerine 0.25 mM. Sonicated chromatin was precipitated with a goat polyclonal anti-IrF4 antibody (M-17; Santa Cruz Biotechnology, Inc.) or control goat IgG (house). Precipitated DNA was assessed by qPCR with the primers indicated in Table S1.

Statistical analysis. Unless otherwise stated, statistical analyses were performed using Prism software. The two-tailed unpaired Student’s t test was used for statistical analysis. When the normality test failed, a Mann-Whitney nonparametric test was performed.

Online supplemental material. Table S1 lists the different primers used in the study; Table S2 lists the different antibodies used in the study for Western blot analysis. Table S3 lists the different antibodies used in the study for flow cytometric analysis. Table S4 describes the number of reads obtained by RNA sequencing for the data presented in Fig 6. Table S5 describes the location of the primers used by CHIP PCR to locate the putative binding of IrF4 in the promoter of Zbhb20. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20131831/DC1.

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