In vivo conditional deletion of HDAC7 reveals its requirement to establish proper B lymphocyte identity and development

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Class IIa histone deacetylase (HDAC) subfamily members are tissue-specific gene repressors with crucial roles in development and differentiation processes. A prominent example is HDAC7, a class IIa HDAC that shows a lymphoid-specific expression pattern within the hematopoietic system. In this study, we explored its potential role in B cell development by generating a conditional knockout mouse model. Our study demonstrates for the first time that HDAC7 deletion dramatically blocks early B cell development and gives rise to a severe lymphopenia in peripheral organs, while also leading to pro–B cell lineage promiscuity. We find that HDAC7 represses myeloid and T lymphocyte genes in B cell progenitors through interaction with myocyte enhancer factor 2C (MEFC2). In B cell progenitors, HDAC7 is recruited to promoters and enhancers of target genes, and its absence leads to increased enrichment of histone active marks. Our results prove that HDAC7 is a bona fide transcriptional repressor essential for B cell development.

INTRODUCTION

Within the adult hematopoietic system, generation of various mature blood cell types is the result of several cell lineage choices and differentiation steps. At each particular branching or differentiation point, the silencing of genes from alternative lineages is crucial for acquiring the correct identity of the newly generated cell. In bone marrow, lymphoid-primed multipotent progenitors commit to the lymphoid branch by generating common lymphoid progenitors, which, in turn, have the ability to give rise to early B and T lymphocyte progenitors (Kondo et al., 1997; Cobaleda and Busslinger, 2008). Once generated, B cell progenitors (pro–B cells) undergo a series of differentiation steps that result in the generation of B cell precursors (pre–B cells) and immature B lymphocytes (Parra, 2009; Barneda-Zahonero et al., 2012). The latter migrate to the spleen to complete their maturation (Parra, 2009; Barneda-Zahonero et al., 2012). Intense research effort has revealed the identity and role of specific transcription factors responsible for the activation of B cell–specific genes (Parra, 2009; Barneda-Zahonero et al., 2012). The transcription factors E2A, EBF, and FOXO1 are involved in the early specification of common lymphoid progenitors into pro–B cells, whereas PAX5 is required to maintain B cell identity during differentiation into mature B cells (Urbánek et al., 1994; Zhuang et al., 1994; Lin and Grosschedl, 1995; Bain et al., 1997; Delogu et al., 2006; Dengler et al., 2008). Recently, Schwickert et al. (2014) reported that IKAROS is also required for early B cell development. In all cases, transcription factors induce the expression of genes characteristic of B cells. Notably, the transcription factor PAX5 not only induces the expression of a B cell–specific genetic program, but also suppresses inappropriate genes of alternative lineages, thereby ensuring proper B cell differentiation (Delogu et al., 2006; Pridans et al., 2008). Likewise, EBF and E2A are also involved in the repression of non–B cell genes (Ikawa et al., 2004; Pongubala et al., 2008; Lukin et al., 2011; Nechanitzky et al., 2013). The transcription factor myocyte enhancer factor 2C (MEF2C) is involved in the commitment of cells to

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Abbreviations used: 7AAD, 7-aminoactinomycin D; ChIP, chromatin immunoprecipitation; HDAC, histone deacetylase; MEF2C, myocyte enhancer factor 2C.

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the lymphoid lineage by activating lymphoid-specific genes and repressing myeloid genes (Stehling-Sun et al., 2009; Kong et al., 2016). How B cell transcription factors induce the silencing of genes that should not be expressed in B cells remains largely unknown.

The large superfamily of histone or protein deacetylases (HDACs) are crucial transcriptional repressors in many physiological and pathological processes. Among them, the class IIa HDAC subfamily, comprising HDAC4, HDAC5, HDAC7, and HDAC9, has specific features that differ from all other HDACs, such as tissue specificity and interaction with transcription factors (Parra and Verdin, 2010; Parra, 2015). Recently, we found that HDAC7 is down-regulated during the in vitro reprogramming of pre–B cells into macrophages, whereas exogenous expression of HDAC7 interfered with the acquisition of essential macrophage-specific cell functions in this in vitro system (Barneda-Zahonero et al., 2013). However, a role for HDAC7 in B cell development in vivo remains to be established. Of interest, Lin et al. (2010) identified *Hdac7* as a target of E2A, EBF, and Foxo1 in B lymphocyte progenitors, whereas Revilla-i-Domingo et al. (2012) showed that *Hdac7* may be a PAX5-activated gene. Whyte et al. (2013) showed that genes involved in cell lineage identity contain superenhancer regions that recruit specific transcription factors leading to their expression. Strikingly, they identified *Hdac7* as one of the lineage identity genes bearing a superenhancer region in pro–B cells (Whyte et al., 2013). These recent studies suggest that HDAC7 may regulate B cell development.

Here, we demonstrate that HDAC7 is essential for proper B cell development. Specifically, HDAC7 deficiency in pro–B cells in mice causes a block in early B cell development. This is accompanied by expression of genes from alternative lineages, such as myeloid cells and T lymphocytes. We propose that HDAC7-mediated repression occurs through MEF2C recruitment to crucial myeloid and T cell genes. These findings establish HDAC7 as a bona fide transcriptional repressor necessary for the acquisition of the correct gene cell identity during B cell generation.

RESULTS AND DISCUSSION

To investigate the potential role of HDAC7 in adult B lymphopoiesis, we generated a conditional mouse model for HDAC7 deficiency in pro–B cells. To this end, we crossed $Hdac7^{loxp/-}$ mice with the deleter line *mb1*-Cre, mb1-$Cre^{ki/+}$ mice (Hobeika et al., 2006). An initial analysis of bone marrow and spleen revealed a 30% and 45% reduction, respectively, in the total number of cells in $Hdac7^{loxp/-}$;mb1-$Cre^{ki/+}$ mice (referred to as $Hdac7^{fl/-}$) compared with their littermate controls $Hdac7^{+/-}$;mb1-$Cre^{ki/+}$ ($Hdac7^{+/-}$ mice; Fig. 1 A). Accordingly, we observed markedly smaller spleens in HDAC7 conditional knockout mice than in their littermate controls (Fig. 1 B). A detailed flow cytometry analysis of the various B cell subsets in the bone marrow showed that HDAC7 deficiency causes a block in B cell development at the pro–B to pre–B cell transition. In particular, we observed a significant decrease in the total number of B cells and an accumulation of pro–B cells in $Hdac7^{fl/-}$ compared with their littermate controls, $Hdac7^{+/-}$ (Fig. 1 C, and see Fig. S1, A–C for gating strategy). In contrast, the number of pre–B cells was significantly lower while immature and mature recirculating B cells were hardly detectable in the bone marrow of HDAC7 mutant mice (Fig. 1 C). Our staining strategy to distinguish between pro–B ($B220^+IgM^-CD43^+$) and pre–B ($B220^+IgM^-CD43^-$) lymphocytes revealed the presence of cells expressing intermediate CD43 levels in bone marrow from HDAC7-deficient mice (see Fig. S1 for gating strategy). A similar CD43 expression pattern was observed when the CD19 marker was included in the analysis (Fig. S1). Furthermore, an alternative staining strategy including CD25 as a marker corroborated that the absence of HDAC7 results in a block in B cell development at the pro–B to pre–B cell transition (Fig. S1). These findings collectively indicate that HDAC7 is necessary for early B cell development.

Next, we assessed the impact of HDAC7 deficiency in peripheral organs. We found that HDAC7 knockout mice had many fewer B cells in the spleen and blood than did control mice (Fig. 1 D). To assess the effect of HDAC7 deficiency at later B cell developmental stages, we next analyzed B cell maturation in the spleen of $Hdac7^{fl/-}$ mice and their littermate controls $Hdac7^{+/-}$. In particular, the analysis of immature B cells, marginal zone B cells, follicular B cells, and transitional B cells revealed that the absolute numbers of all B cell subtypes analyzed were significantly lower in HDAC7-deficient mice (Fig. 1 D, and see Fig. S2 for gating strategy). Hematoxylin and eosin staining revealed that the spleens of control mice had a normal structure with well-defined follicles represented by white pulp (hematoxylin staining in purple; Fig. 1 E) surrounded by red pulp (eosin staining in pink; Fig. 1 E). We observed that spleens from HDAC7-deficient mice had a highly abnormal and unstructured morphology, with smaller and poorly defined follicles (Fig. 1 E). We examined whether the proportions of macrophages and T cells were altered in the spleens of HDAC7 knockout mice. We found significantly higher proportions of Mac-1–positive cells in the spleens of HDAC7 mutant mice (Fig. 1 F). In addition, flow cytometry analysis revealed a higher percentage of T lymphocytes in HDAC7 knockout mice (Fig. 1 G). The data presented so far demonstrate that HDAC7 is essential for proper B lymphocyte development and its absence is associated with severe lymphopenia in the periphery and a higher density of macrophages and T cells in the spleen.

To get an insight into the mechanisms underlying the B lymphocyte developmental block observed, we first determined whether the absence of HDAC7 affected cell proliferation. Cell cycle analysis revealed that proliferation was not sensibly altered in either pro–B or pre–B cell subsets in the absence of HDAC7 (Fig. 2 A). Likewise, we did not observe differences in the expression of *Il7r* in pro–B or pre–B cells from wild-type and HDAC7-deficient mice, thus rein-

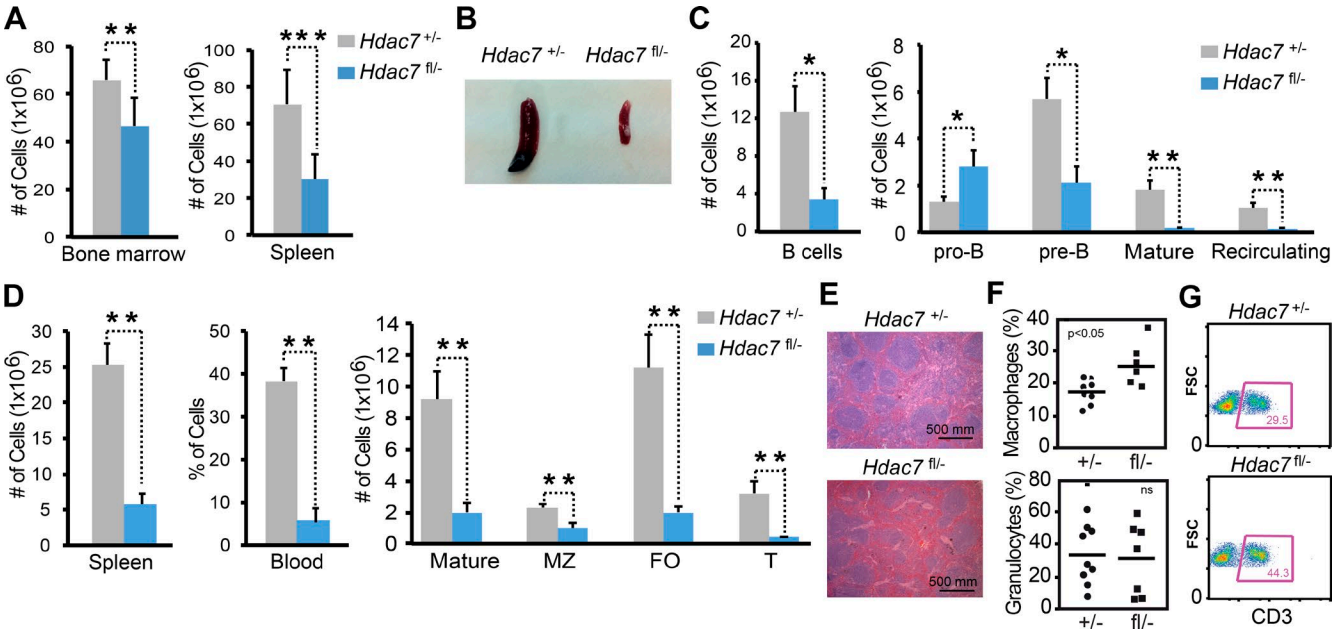

Figure 1. **HDAC7 is required for early B cell development.** (A) Absolute numbers of total bone marrow cells (*Hdac7*$^{fl/-}$ [*n* = 6] and *Hdac7*$^{+/-}$ [*n* = 8]) and total spleen cells (*Hdac7*$^{+/-}$ [*n* = 12] and *Hdac7*$^{fl/-}$ [*n* = 9]). (B) Representative photograph of the spleen from *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ mice. (C) Absolute numbers of bone marrow B220^{+} B cells (*Hdac7*$^{fl/-}$ [*n* = 5] and *Hdac7*$^{+/-}$ [*n* = 8]), B220^{+}CD43^{+}IgM^{-} pro-B cells (*Hdac7*$^{fl/-}$ [*n* = 5] and *Hdac7*$^{+/-}$ [*n* = 8]), B220^{+}CD43^{-}IgM^{-} pre-B cells (*Hdac7*$^{fl/-}$ [*n* = 6] and *Hdac7*$^{+/-}$ [*n* = 6]), B220^{+}IgM^{+} immature B cells (*Hdac7*$^{fl/-}$ [*n* = 7] and *Hdac7*$^{+/-}$ [*n* = 9]), and B220^{+}IgM^{+} IgD^{+} mature recirculating B cells (*Hdac7*$^{fl/-}$ [*n* = 7] and *Hdac7*$^{+/-}$ [*n* = 9]). (D) Graph on the left shows the absolute numbers of B220^{+} B cells from spleen of *Hdac7*$^{+/-}$ (*n* = 12) and *Hdac7*$^{fl/-}$ (*n* = 9) mice. Graph in the middle shows the percentage of B220^{+} cells in the blood from *Hdac7*$^{+/-}$ (*n* = 4) and *Hdac7*$^{fl/-}$ (*n* = 4) mice. Graph on the right shows absolute numbers of spleen B cell subsets from wild-type and HDAC7-deficient mice: B220^{+}IgM^{+}IgD^{+} mature B cells (*Hdac7*$^{+/-}$ [*n* = 7] and *Hdac7*$^{fl/-}$ [*n* = 5]), CD21^{bright}CD23^{+} marginal zone (MZ) B cells (*Hdac7*$^{+/-}$ [*n* = 12] and *Hdac7*$^{fl/-}$ [*n* = 9]), and CD21^{+}CD23^{bright}CD93^{-} follicular (FO; *Hdac7*$^{+/-}$ [*n* = 9] and *Hdac7*$^{fl/-}$ [*n* = 7]) and CD21^{+}CD23^{bright}CD93^{+} transitional (T; *Hdac7*$^{+/-}$ [*n* = 8] and *Hdac7*$^{fl/-}$ [*n* = 7]) B cells. (E) Hematoxylin and eosin staining of the spleen from *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ mice. (F) Percentages of granulocytes and macrophages from the spleen of *Hdac7*$^{+/-}$ (*n* = 9) and *Hdac7*$^{fl/-}$ (*n* = 7) mice. Mean values are shown as horizontal bars. ns, not significant. (G) Representative FACS analyses from three independent experiments are shown on T lymphocytes from the spleens of *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ mice. FSC, forward scatter. Data are represented as the mean ± SEM. Statistical significances were identified using the unpaired two-tailed Student's *t* test. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

forcing the finding that the proliferation status is intact in HDAC7-deficient B cells (Fig. 2 B). Therefore, we wondered whether the developmental block at the pro–B to pre–B cell transition in mice lacking HDAC7 might be associated with cell death. HDAC7-deficient pre–B cells showed a significant increase of 7-aminoactinomycin D (7AAD)$^{+}$ cells compared with wild-type pre–B cells (Fig. 2 C). We also observed a trend to an increased cell death rate in HDAC7-deficient pro–B cells. Next, to test whether HDAC7 is required for V(D)J recombination in pro–B and pre–B cells, we examined the expression of intracellular IgHμ protein. We found that HDAC7-deficient pro–B and pre–B cells express IgHμ (although to a significantly lesser extent than control cells; Fig. 2 D), indicating that HDAC7 is not absolutely required for V(D)J recombination. Western blot experiments revealed that the Cre-Loxp system used in our study was highly efficient because HDAC7 was completely absent in B cells from bone marrow of knockout mice (Fig. 3 G). Therefore, V(D)J rearrangements in pro–B and pre–B cells from our knockout mouse model are not likely caused by the presence of residual HDAC7. Together, these data indicate that pro–B and pre–B cells lacking HDAC7 undergo V(D)J recombination but are more susceptible to apoptosis, which may explain the severely reduced numbers of pre–B and subsequent B cell subsets in the bone marrow and the periphery of HDAC7-deficient mice.

Because HDAC7 is a bona fide transcriptional repressor, we wondered whether HDAC7 is involved in silencing lineage-inappropriate genes, thereby ensuring the correct acquisition of B cell identity. To address this possibility, we performed global gene expression profiling in pro–B cells purified from *Hdac7*$^{fl/-}$ mice and their *Hdac7*$^{+/-}$ littermate control mice. Microarray analysis showed that 1,750 and 1,424 genes were up-regulated and down-regulated, respectively, in HDAC7-deficient pro–B cells (Dataset S1). To gain functional insights into the genes that were up-regulated in the absence of HDAC7, we performed gene set enrichment analysis based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and the gene ontology (GO) categories corresponding to Biological Processes (Fig. 3 A). The KEGG pathway analysis showed that the up-regulated genes were enriched in MAPK signaling, T cell receptor signaling, lysozyme, ubiquitin-mediated proteolysis, endo-

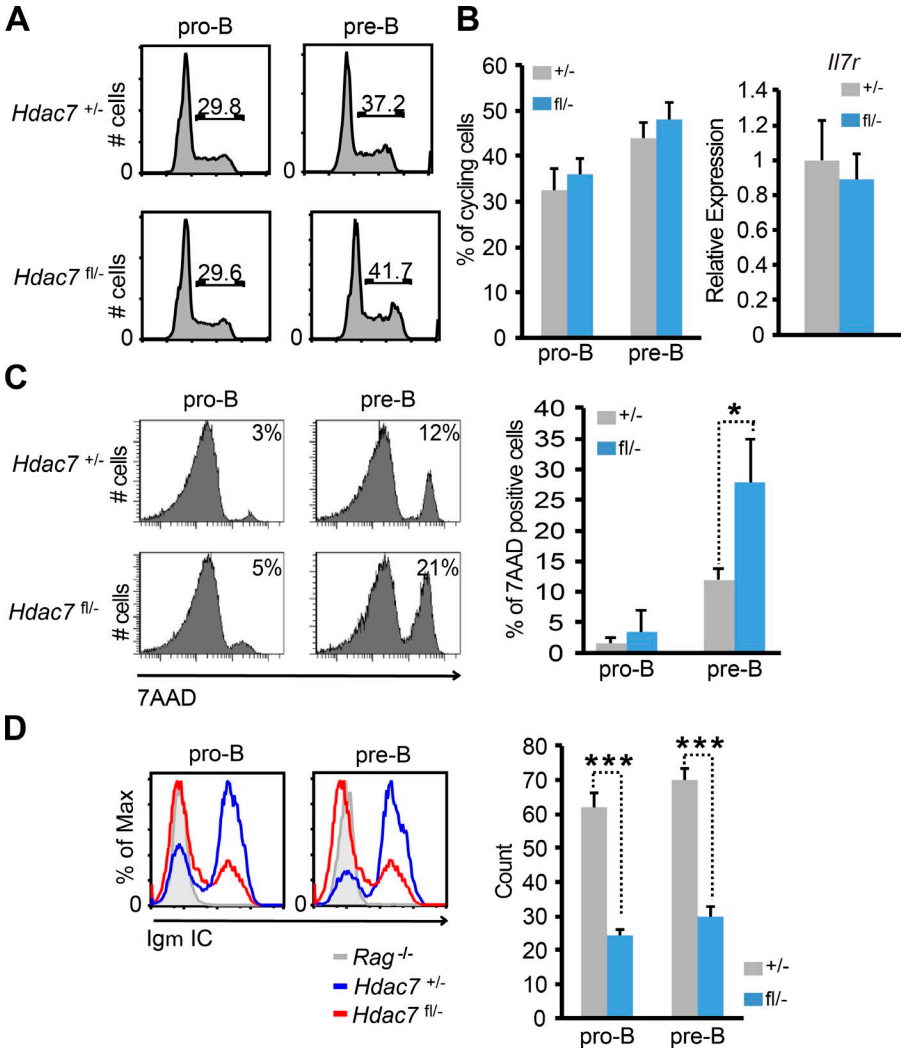

Figure 2. **HDAC7 deficiency results in increased cell death in pro–B and pre–B cells.** (A) Representative Hoechst staining of pro–B and pre–B cells from *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ mice form three independent experiments. Bars represent the percentage of cycling cells. (B) RT-quantitative PCR experiments for gene expression of *Il7r* in pro–B cells from *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ mice. (C) Increased cell death of pro–B and pre–B cells in the absence of HDAC7. Bar graph depicts the percentage of apoptotic-dead (7AAD^{+}) cells. (D) Representative histogram staining showing the expression of intracellular IgHμ (Igm IC) in pro–B and pre–B cells from *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ mice. Bar graph depicts the cell count expressing IgHμ. All data are represented as the mean ± SEM of three independent experiments. Statistical significances were identified using the unpaired two-tailed Student's *t* test. *, $P < 0.05$; ***, $P < 0.001$.

cytosis, chemokine signaling, and Fc-γ–mediated phagocytosis pathways (Fig. 3 A). The Biological Processes analysis revealed that the up-regulated genes belong to the categories representing transcription, chromatin organization and modification, hemopoiesis, leukocyte activation, intracellular signaling cascade, immune system development, endocytosis, T cell activation and differentiation, and myeloid activation processes (Fig. 3 A). Strikingly, we observed that the absence of HDAC7 from pro–B cells was associated with the up-regulation of key genes for myeloid cell functions, such as *Itgam*, *Itgax*, *Ifi204*, *Ccl3*, *Ccl4*, and *Ccr2*, among others (Table S1). Indeed, HDAC7 deficiency prompted the expression of the key macrophage marker Mac-1 (encoded by *Itgam*) in gated bone marrow lymphocytes or purified CD19^{+} B cells (Fig. 3, B and C). In addition, loss of HDAC7 from pro–B cells led to the up-regulation of many transcription factors, several of which play a role in myeloid cell differentiation (*Fosb*, *Egr1*, *Crebzf*, *Cebpb*, and *Cebpd*; Table S1). Strikingly, analysis of data from the Immunological Genome Project to compare the expression pattern between HDAC7 and selected myeloid genes in purified B cells and macrophage populations revealed an inverse correlation with gene expression (Fig. 3 D). We also found that HDAC7 deficiency led to the up-regulation of T cell genes, such as *Cd28*, *Cd69*, *Il17*, and *Lck*, and the T cell transcription factors *Runx1*, *Runx3*, and *Nfat5* (Table S1). Changes in the expression of selected genes (*Itgam*, *Ccl3*, *Cd28*, and *Cd69*) were validated by quantitative RT-PCR (Fig. 3 E). Interestingly, analysis of the down-regulated genes in the absence of HDAC7 did not reveal any enrichment in either gene ontology or KEGG pathways related to biological aspects of B lymphocytes (not depicted). Of note, our microarray experiments did not reveal any changes either in the expression of key B cell transcription factors such as PAX5, E2A, and EBF or in other important B lymphocyte genes (CD19 and RAG2). The expression of *Cd19*, *Pax5*, and *Rag2* was determined by quantitative RT-PCR (Fig. 3 F). PAX5 and EBF protein levels were determined by Western blot assays (Fig. 3 G). The fact that HDAC7 deficiency imposes a strong block on B cell development, even in the presence of B cell master regulators, indicates that it is a potent regulator of

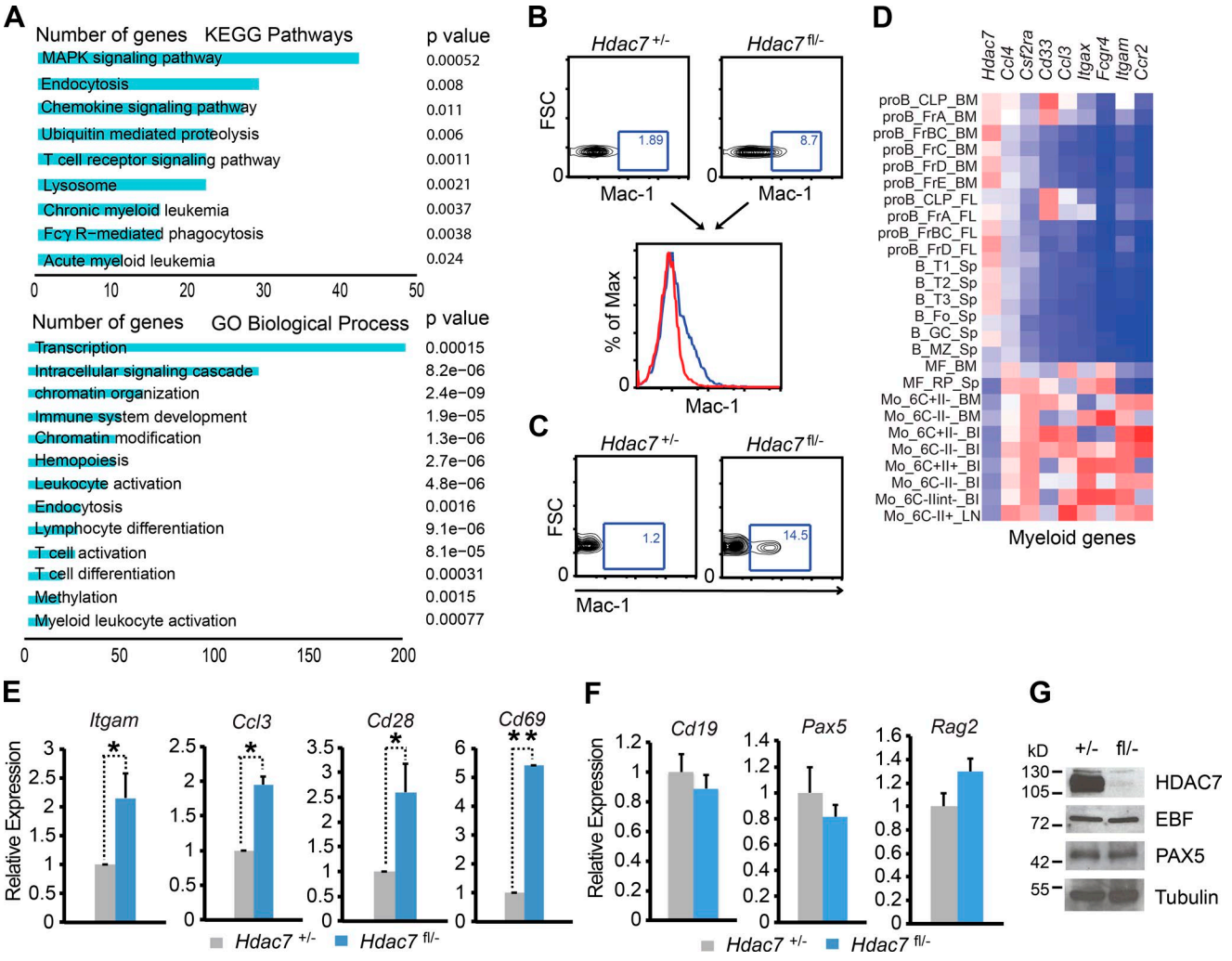

Figure 3. **Pro–B cells from HDAC7-deficient mice express genes from alternative lineages.** (A) KEGG pathway enrichment and Gene Ontology (GO) analysis of up-regulated genes in HDAC7-deficient pro–B cells. (B) Representative FACS analyses from three independent experiments showing Mac-1 expression in gated bone marrow lymphocytes from *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ mice (top). Histogram showing Mac-1 expression in gated bone marrow lymphocytes from *Hdac7*$^{+/-}$ (red) and *Hdac7*$^{fl/-}$ (blue) mice (bottom). FSC, forward scatter. (C) Representative FACS analyses from three independent experiments showing Mac-1 expression in purified CD19^{+} bone marrow lymphocytes from *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ mice. (D) Heatmap showing the expression pattern of HDAC7 and selected myeloid genes in different hematopoietic cell subsets. Data were obtained from the Immunological Genome Project. A description of the different cell subsets analyzed can be found at www.Immgen.org. (E) RT–quantitative PCR experiments for gene expression changes for selected up-regulated genes in the absence of HDAC7. *, $P < 0.05$; **, $P < 0.01$. (F) RT–quantitative PCR experiments for gene expression levels of B cell genes. (E and F) Data are represented as the mean ± SEM of three independent experiments. (G) Western blot analysis for the expression of HDAC7 and B cell transcription factors in wild-type and HDAC7-deficient B lymphocytes.

B cell lymphopoiesis. Collectively, these findings demonstrate that HDAC7 acts as a transcriptional repressor of lineage-inappropriate genes in B cell progenitors.

Because we previously showed that HDAC7 specifically interacts with the transcription factor MEF2C but not with other B cell–specific transcription factors in B cell precursors (Barneda-Zahonero et al., 2013), we examined whether HDAC7 is recruited to MEF2 binding sites located at the promoter of nonlymphoid genes in pro–B cells, leading to their transcriptional silencing, by performing chromatin immunoprecipitation (ChIP) experiments. First, using the TFconsite bioinformatic tool, we found that promoters of *Itgam*, *Cd69*, *Cd28*, and *Ccl3* contain putative MEF2 binding sites. Chromatin prepared from bone marrow wild-type B cells was subjected to ChIP assays with an antibody specific to HDAC7. Quantitative PCR analysis of the immunoprecipitated material with specific primers for the *Itgam*, *Ccl3*, *Cd28*, and *Cd69* promoters indicated that HDAC7 was enriched at their putative binding sites in these loci in pro–B cells (Fig. 4 A). Interestingly, we did not observe an

enrichment of HDAC7 at the *Pax5* promoter (Fig. 4 A). We also found that MEF2C was enriched at the promoters of HDAC7 target genes (Fig. 4 A). To definitively prove that HDAC7 represses its target genes through interaction with MEF2C, we performed a rescue or gain of function experimental approach. We transduced purified B cells from the bone marrow and spleens of wild-type and HDAC7-deficient mice with retroviral vectors for either normal HDAC7 or a mutant form carrying a deletion of the entire 17–amino acid stretch that mediates the interaction with MEF2 transcription factors (MIG-HDAC7-ΔMEF; Dequiedt et al., 2003), fused to GFP, and GFP-positive cells were sorted. We tested the HDAC7 mutant for its ability to repress *Itgam* and *Cd69* in bone marrow B cells lacking HDAC7. As experimental controls, we transduced wild-type and HDAC7-deficient cells with empty retroviral vector (MIG). Expression of wild-type HDAC7 in knockout cells resulted in a significant decrease in the expression of *Itgam* and *Cd69* mRNA levels (Fig. 4 B), whereas expression of the HDAC7 mutant defective for MEF2C binding had no significant effect. We also observed that the expression of wild-type, but not mutant, HDAC7 reduced the expression of Mac-1 protein in purified B cells in the spleens of knockout mice (Fig. 4 C). These experiments demonstrate that the HDAC7–MEF2C interaction is necessary for HDAC7 to repress its target genes.

Next, we analyzed the enrichment of active and repressive histone marks at HDAC7 target genes in the absence or presence of HDAC7. Interestingly, we observed that H3Ac(K9/K14) and H4K16Ac were already present at the promoters of HDAC7 target genes in wild-type B cells. The absence of HDAC7 from B cells was associated with a moderate but significant increase of both histone marks and a decrease of the repressive mark H3K27me3 in the promoters of its target genes (Fig. 4 D). To test whether, in addition to target promoters, H3Ac(K9/14) are also enriched at other genomic regions, we performed ChIP-seq assays using purified pro–B cells from wild-type and HDAC7-deficient mice. Both histone marks were also found at other genomic locations such as introns, exons, and intergenic regions (Fig. 5 A and Dataset S2). Interestingly, many of the genes up-regulated in HDAC7-deficient pro–B cells showed an enrichment in H3Ac(K9/K14) in wild-type and HDAC7-deficient pro–B cells (Dataset S3). At the global level, only slight changes in enrichment of H3Ac(K9/K14) were detectable in the absence of HDAC7 (Fig. 5 B). However, individual analysis by ChIP coupled with quantitative PCR confirmed the significance of such changes observed for HDAC7-deficient pro–B cells (Fig. 4 D). Karmodiya et al. (2012) recently reported that H3K9Ac and H3K14Ac marks not only associate with promoters of actively transcribed genes, but also with developmentally regulated bivalent promoters, as well as with enhancers in mouse embryonic stem cells. Similarly, pro–B cells in the bone marrow are largely undifferentiated lymphocyte progenitors, and the presence of histone active marks may indicate that genes from alternative lineages are poised, resulting in a certain degree of cell plasticity toward other hematopoietic cell types. In fact, genetic ablation of key B cell factors such as PAX5, E2A, and EBF1 in mice results in the inappropriate expression of genes from other cell types within the hematopoietic system. Accordingly, here we demonstrate that HDAC7 deficiency from pro–B cells leads to expression of myeloid and T cell genes. Recently, van Oevelen et al. (2015) determined the presence of enhancers at macrophage genes in pre–B cells. The authors showed that during trans-differentiation of pre–B cells into macrophages by exogenous expression of the myeloid transcription factor C/EBP-α, this transcription factor binds to two types of myeloid enhancers in B cells: preexisting enhancers that are bound by PU.1, providing a platform for incoming C/EBP-α; and de novo enhancers that are targeted by C/EBP-α (van Oevelen et al., 2015). This prompted us to analyze whether these macrophage gene enhancers were also enriched in H3K9/K14Ac in our ChIP-seq experiment. Interestingly, preexisting enhancers that are bound by PU.1 were indeed occupied by both histone marks in wild-type and HDAC7-deficient pro–B cells (Fig. 5 B and Dataset S4). This finding further supports the notion that lineage-inappropriate genes may be epigenetically poised in B cell progenitors. To test the possibility that HDAC7 could be also recruited at enhancers of its target genes, we performed ChIP experiments. Strikingly, we found that HDAC7 is also bound to enhancers of *Itgam* and *Cd69* (Fig. 5 C), in addition to their promoters. The absence of HDAC7 from pro–B cells was associated with a significant enrichment of the activating histone marks, H3Ac(K9/K14) and H4K16Ac, in the enhancers of its target genes. Interestingly, enrichment of the enhancer mark H3K27Ac was also increased in the same enhancer loci in HDAC7-deficient pro–B cells (Fig. 5 D). Collectively, our results demonstrate that HDAC7 is recruited to the promoters and enhancers of lineage-inappropriate genes in pro–B cells, resulting in their transcriptional silencing. The finding that HDAC7 is also recruited at enhancers of its target genes represents a novel mechanism by which this HDAC may control gene expression in B lymphocytes.

Our results represent the first evidence that HDAC7 acts in vivo as a master regulator of B cell identity and development. We demonstrate that HDAC7 is an essential transcriptional repressor of genes from alternative lineages that ensures proper B cell development. The fact that B cell transcription factors, such as E2A and PAX5, may induce the expression of HDAC7 in pro–B cells indicates the potential existence of an alternative mechanism involved in gene repression. B cell transcription factors not only recruit corepressors to silence their lineage-inappropriate target genes, but also may act through an indirect mechanism that induces the expression of a transcriptional repressor, such as HDAC7, which, in turn, through the interaction with MEF2C, directly represses genes from alternative lineages. In conclusion, we have identified HDAC7 as a lymphoid-specific and bona fide transcriptional repressor that is essential for proper B lymphocyte development and for ensuring the acquisition of the correct gene

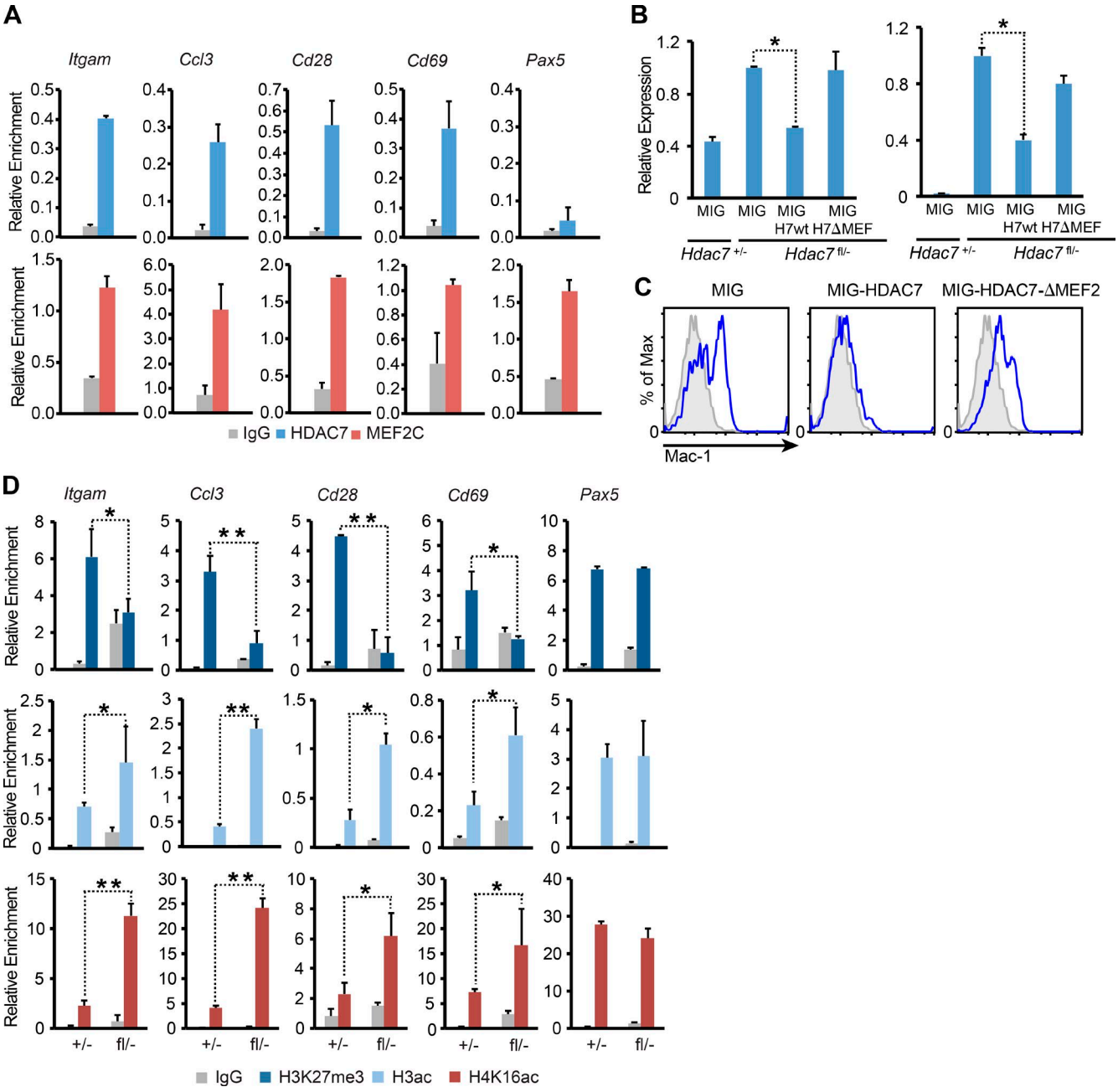

Figure 4. **HDAC7 and MEF2C are recruited to the promoters of lineage-inappropriate genes in bone marrow B lymphocytes.** (A) ChIP experiments showing the enrichment of HDAC7 and MEF2C to putative MEF2 binding sites on the *Itgam*, *Ccl3*, *Cd28*, *Cd69*, and *Pax5* gene loci in bone marrow CD19$^+$ B cells. Results are presented as the relative enrichment over input and are based on the results of three independent experiments. (B and C) Purified HDAC7-deficient B cells from bone marrow (B) and spleen (C) were infected with MSCV-GFP, MSCV-GFP-HDAC7, and MSCV-GFP-HDAC7(ΔMEF) viruses. As an experimental control, wild-type B cells were transduced with MSCV-GFP. (B) GFP$^+$ cells were sorted and mRNA extracted. RT–quantitative PCR experiments for *Itgam* (left) and *Cd69* (right) gene expression changes in HDAC7-deficient B cells. Data are given as mean ± SEM of values obtained in three independent experiments. (C) Cells were stained with a Mac-1 antibody, and the GFP-positive fractions were gated and the results plotted. (D) ChIP experiments showing the enrichment of H3Ac(K9/K14), H3K27me3, and H4K16Ac to the *Itgam*, *Ccl3*, *Cd28*, *Cd69*, and *Pax5* gene loci in bone marrow CD19$^+$ B cells from *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ mice. Results are presented as the relative enrichment over input and are based on the results of three independent experiments. Data are given as mean ± SEM of values obtained in three independent experiments. Statistical significances were identified using the unpaired two-tailed Student's *t* test. *, $P < 0.05$; **, $P < 0.01$.

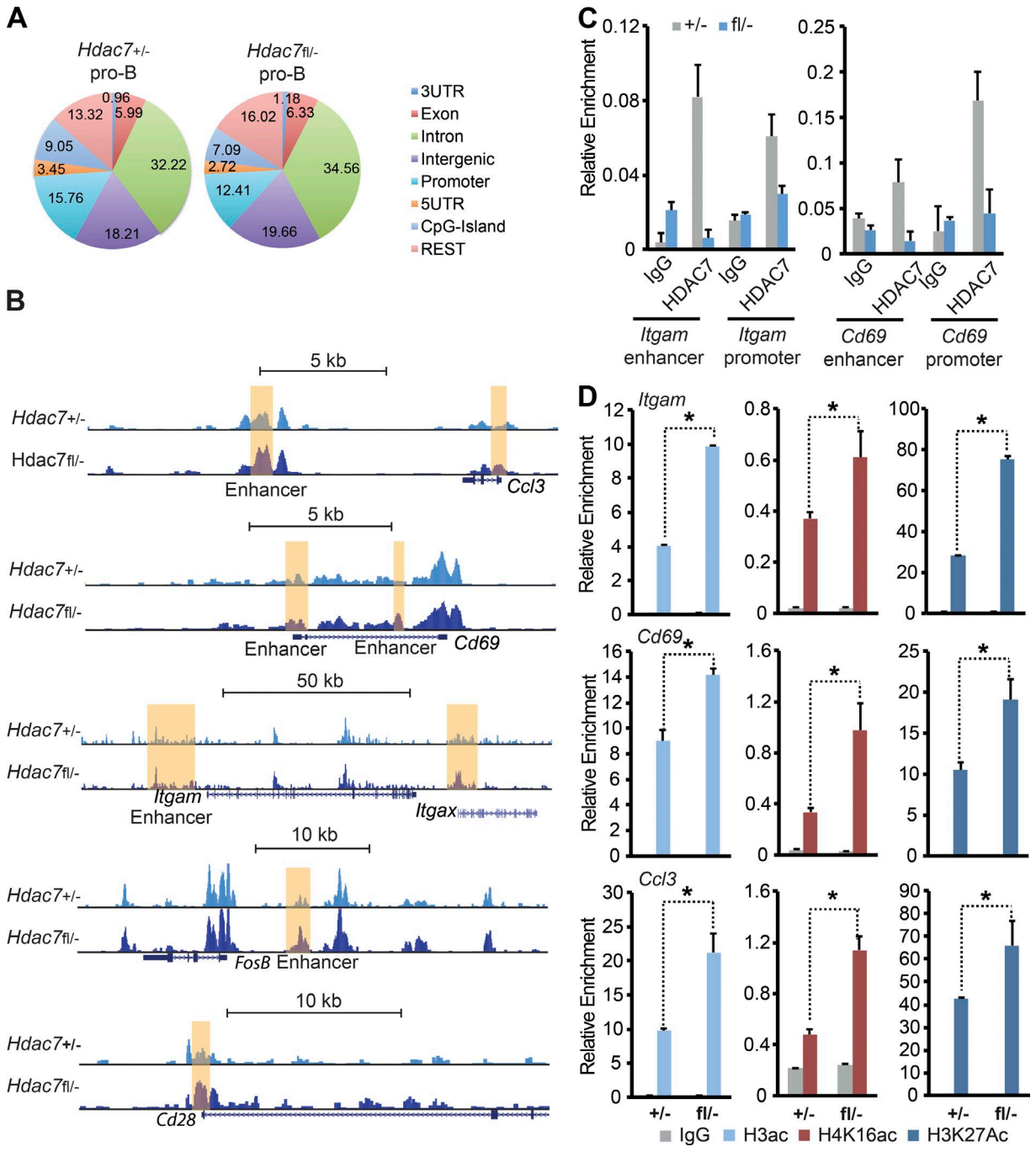

Figure 5. **HDAC7 is recruited at enhancers of its target genes, and its deficiency results in increased enrichment of histone active marks.** (A) Genomic distribution of H3Ac(K9/K14) enrichment in *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ pro-B cells. (B) Examples for H3Ac(K9/K14) enrichment at enhancers and promoters of selected genes. (C) ChIP experiments showing the enrichment of HDAC7 to *Itgam* and *Cd69* promoters and enhancers in pro-B cells from *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ mice. (D) H3Ac(K9/K14), H4K16Ac, and H3K27Ac enrichment to the *Itgam*, *Ccl3*, and *Cd69* enhancers in pro-B cells from *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ mice. Results are presented as the relative enrichment over input and are based on the results of three independent experiments. (C and D) Data are given as mean ± SEM of values obtained in three (C) and four (D) independent experiments. Statistical significances were identified using the unpaired two-tailed Student's *t* test. *, $P < 0.05$.

identity of B cells. Our findings represent a significant advance in our understanding of the transcriptional complexity underlying B cell generation.

MATERIALS AND METHODS

Mouse and animal care

Hdac7$^{fl/-}$ mice were previously described (Chang et al., 2006) and were provided by E. Olson (University of Texas Southwestern Medical Center, Dallas, TX). mb1-Cre$^{ki/+}$ mice were provided by M. Reth (Max Planck Institute of Immunology and Epigenetics, Freiburg, Germany). Experiments were performed with mice that were 4–6 wk of age. Animal housing and handling, and all procedures involving mice, were approved by the Bellvitge Biomedical Research Institute ethics committee, in accordance with Spanish national guidelines and regulations.

Flow cytometry and cell-sorting experiments

Cells were extracted from bone marrow and spleen. Isolated cells were incubated with Fc receptor–blocking antibody (BD) for 10 min at 4°C to reduce nonspecific staining. Cells were then stained with anti-B220 (PerCP-Cy5.5), anti-CD43 (APC), anti-IgM (FITC), anti-CD25 (APC), anti-IgD (PE), anti-Gr1 (PE), anti-CD11b (APC), anti-CD21 (FITC), anti-CD23 (PE), anti-CD93 (APC), and anti-CD3 (FITC; BD) for 30 min at 4°C in the dark. For intracellular IgHμ staining, cells were first stained with surface markers, permeabilized and fixed with buffer Perm/Wash (BD), and stained with anti-IgM mu-biotin antibody (Jackson ImmunoResearch Laboratories, Inc.) followed by incubation with streptavidin-PE. Cells were processed in a Gallios flow cytometer (Beckman Coulter), and the data were analyzed using FlowJo software (Tree Star). For cell-sorting experiments, bone marrow cells were incubated with Fc receptor–binding antibody and then stained with anti-B220 (PerCP-Cy5.5), anti-CD43 (APC), and anti-IgM (FITC; BD), under the same conditions. B220$^+$CD43$^+$IgM$^-$ cells were isolated on a MoFlo sorter (Beckman Coulter).

Retroviral supernatant generation and cellular transduction

For retrovirus generation, the MSCV-GFP, MSCV-GFP-HDAC7, and MSCV-GFP-HDAC7(ΔMEF) plasmids were transfected into the packaged cell line Platinum-E, and supernatants were collected at 48–72 h after transfection. Purified B cells were spin infected, and 48 h later, GFP$^+$ cells were either sorted or analyzed by flow cytometry.

Spleen section histology

Spleens from *Hdac7*$^{fl/-}$ mice and *Hdac7*$^{+/-}$ control mice were fixed in 4% formaldehyde, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin. Samples were imaged under a Scope.A1 microscope (ZEISS).

Western blot

Western blot analysis was performed according to standard procedures. Western blots were developed with the Enhanced Chemiluminescence detection kit (GE Healthcare).

Proliferation and cell cycle assays

Cell proliferation and cell death were assessed by Hoechst and 7AAD staining, respectively. Cells were analyzed by flow cytometry using a Gallios flow cytometer.

Microarray experiments

Total RNA from sorted pro–B cells of *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ mice was extracted by TRIzol and then purified. PCR-amplified RNAs were hybridized against mouse array chips (Mouse Genome 430 p.m. strip; Affymetrix) at the Institute for Research in Biomedicine Genomics Facility (Barcelona, Spain). Microarray analysis (GEO accession no. GSE66163) was performed as previously described (Barneda-Zahonero et al., 2013).

RT–quantitative PCR assays

Pro–B cells were purified by cell sorting. RNA was extracted by TRIzol extraction (QIAGEN), and cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). RT–quantitative PCR was performed in triplicate using SYBR Green I Master (Roche). PCR reactions were run and analyzed using the LightCycler 480 Detection System (Roche).

ChIP assays

For ChIP assays, purified CD19$^+$ B or pro–B cells from the bone marrow of *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ mice were cross-linked with 1% formaldehyde and subjected to immunoprecipitation after sonication. ChIP experiments were performed using the LowCell# ChIP kit (Diagenode) according to the manufacturer's instructions. The following antibodies were used: anti-HDAC7 (Abcam), anti-MEF2C (Cell Signaling Technology), anti-H3Ac(K9/K14) (EMD Millipore), anti-H4K16Ac (Active Motif), anti-H3K27me3 (EMD Millipore), and anti-H3K27Ac (Abcam). Analyses were performed by real-time quantitative PCR. Data are represented as the ratio between the bound fraction of the HDAC7, MEF2C, and histone modification antibody relative to the input control.

ChIP-seq experiments

Sorted pro–B cells from the bone marrow of wild-type and *Hdac7*-deficient mice were cross-linked with 1% formaldehyde and sonicated using an S220 Focused ultrasonicator (Covaris). An anti-H3Ac(K9/K14) antibody (EMD Millipore) was used to perform ChIP from 100,000 pro–B cells using the True MicroChIP kit (Diagenode) according to the manufacturer's instructions. ChIP library construction and sequencing were performed according to standard procedures at the Centre for Genomic Regulation Genomics Core Facility (Barcelona, Spain). The quality of raw reads was checked using FastQC, and adapters were trimmed with a Skewer trimming tool (Bioinformatics) before mapping. Trimmed reads were mapped against the mouse genome (*Mus musculus*, genome version mm9) with Burrows-Wheeler Aligner–MEM, and duplicates were removed with the MarkDuplicates

tool in the Picard software. Data analysis, involving peak calling and annotation, was performed by qGenomics Laboratories using the Hypergeometric Optimization of Motif EnRichment v.4.8 suite. Variable-width peaks were called in the immunoprecipitated sample, using the input sample as a control, with the findPeaks function in the histone mode and a false discovery rate set to 0.001. Peaks were annotated using the annotatePeaks (Integrative Genomics) tool (SRA accession no. SRP076788).

Statistics

Statistical significance was determined by the two-tailed unpaired Student's *t* test.

Online supplemental material

Figs. S1 and S2 show the gating strategy used in the phenotypic characterization of wild-type and HDAC7-deficient mice. Table S1 shows a selection of up-regulated genes in HDAC7-deficient pro–B cells. Dataset S1 shows the list of genes up-regulated and down-regulated in HDAC7-deficient pro–B cells. Datasets S2, S3, and S4 show the ChIP-seq analysis of H3Ac(K9/K14) enrichment in wild-type and HDAC7-deficient pro–B cells (genomic distribution, annotated peaks, and enrichment at macrophage gene enhancers).

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