Abbreviations used: ASC, antibody-secreting cells; ChIP, chromatin immunoprecipitation; CSR, class switch recombination; DEG, di erentially expressed genes; GC, germinal center; miRNA, microRNA; RPKM, reads per transcript corrected per million mapped reads; Seq, sequencing; UTR,

The study of the regulatory networks that control cell fate decisions and developmental processes in mammals has mainly been focused on identifying the molecular components and their interactions, usually in a qualitative rather than a quantitative manner. A successful example of this approach is the well-characterized system of terminal di erentiation of B cells, which allows study of the interconnected processes of cellular expansion, di erentiation, and cell fate determination. Antigen-activated B cells receive additional signals from helper T cells before undergoing proliferative expansion. After a few rounds of division, some of the resulting B-blasts migrate to the extrafollicular regions in the spleen or to the medullary cords of lymph nodes, where they continue to proliferate before di erentiating into antibody-secreting cells (ASCs; the term is used here to include cycling plasmablasts and plasma cells). This leads to the immediate production of neutralizing antibody that can be critical to the control of the spread of an infection as well as to the formation of immune complexes that assist antigen presentation (MacLennan et al., 2003; Belver et al., 2011). Such extrafollicular responses can involve antibody (Ab) class switch recombination (CSR) to various isotypes, allowing the Abs produced to acquire a wide range of e ector functions and to disseminate toward infected tissues. Other B-blasts migrate to the B cell follicles, make cognate interactions with antigen-primed T cells and form germinal centers (GC). After accumulation of somatic mutations in their immunoglobulin genes, GC-B cells are subjected to antigen a nity-based selection. This process shapes the BCR repertoire of antigen experienced B cells by providing survival signals to non self-reactive, high a nity clones to become long-lived plasma cells or memory B cells (Ho et al., 1986; Jacob et al., 1991a; Liu et al., 1991).

B cell terminal di erentiation is a particularly attractive system in which to study gene regulatory networks because of the well-de ned gene expression changes that occur during the progression from naive B cells to ASCs and the

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a negative regulator of CSR (Vigorito et al., 2007). Third, PU.1 expression in B cells is elevated in the absence of miR-155, a miRNA that regulates T cell–dependent antibody responses in a B cell intrinsic manner (Vigorito et al., 2007). Last, the role of PU.1 in terminal B cell di erentiation is only beginning to be understood (Carotta et al., 2010b), so our investigation might yield valuable new insights into the mechanisms of this process.

By removing the miR-155–binding site in the 3

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signi cance of this interaction in vivo. We took advantage of our previous observations with miR-155 mice in which we demonstrated a B cell–intrinsic defect in the T cell–dependent response to NP-KLH (Vigorito et al., 2007). We started by measuring the level of steady-state serum immunoglobulins

Figure 2. PU.1 is a negative regulator of Ig secretion in vivo. (A)

of the extrafollicular response but also uncover a novel inhibitory role of PU.1 in terminal B cell di erentiation in vivo. Our results also suggest that the amount of PU.1 is under stringent control in vivo, and small changes in its expression, due to miRNA regulation, a ect adaptive immune responses.

Impaired plasma cell differentiation in activated PU.1$^{155/155}$ B cells correlates with increased e pression of Pa 5

To assess CSR and plasma cell di erentiation independently of cell proliferation (Hasbold et al., 2004), we labeled B cells

with 5-(6) CFSE and examined cell surface expression of IgG1 and CD138 (a marker of plasmablasts and plasma cells) by ow cytometry over a time course of 5 d after stimulation with LPS and IL4. Cell division in PU.1$^{155/155}$ B cells was not di erent from that in WT B cells (Fig. 3, A and C). It was also not grossly a ected in miR-155$^{/}$ B cells, as shown here (Fig. 3, A and C) and in previous studies (Thai et al., 2007; Dorsett et al., 2008; Teng et al., 2008). In contrast, CSR and plasma cell di erentiation were signi cantly reduced both in PU.1$^{155/155}$ and miR-155–de cient B cells at all time points examined (Fig. 3 B). These observations suggest a developmental defect independent of cell cycle. Consistent with this, we previously showed intact post-switch circle transcription in miR-155$^{/}$ B cells compared with WT (Vigorito et al., 2007). Disruption of the miR-155–binding site in *Aicda* results in enhanced class switching by B cells (Dorsett et al., 2008; Teng et al., 2008), indicating that other miR-155 targets, unlike PU.1, are enhancers of class switching. Our results establish PU.1 as a consequential target of miR-155 that inhibits CSR and plasma cell di erentiation.

Conditional deletion of Blimp1 in B cells has revealed that the plasma cell di erentiation program is initiated by down-regulation of Pax5, which is followed by up-regulation of Blimp1 (Kallies et al., 2007), although it is still unclear how down-regulation of Pax5 is achieved. Expression of Pax5 in B cells is dependent on a promoter region regulated by EBF1 and a potent enhancer in its intron 5 (Decker et al., 2009). Interestingly, in early B cell development, the activity of this enhancer is regulated by the transcription factors PU.1, IRF4, IRF8, and NF-KB (Decker et al., 2009). We therefore hypothesized that the inhibitory e ect of PU.1 on plasma cell di erentiation is caused by a failure of PU.1$^{155/155}$ B cells to down-regulate Pax5. We rst tested whether PU.1 binding to the Pax5 enhancer is detectable in activated B cells and whether that binding is a ected by PU.1 abundance. b4B>T

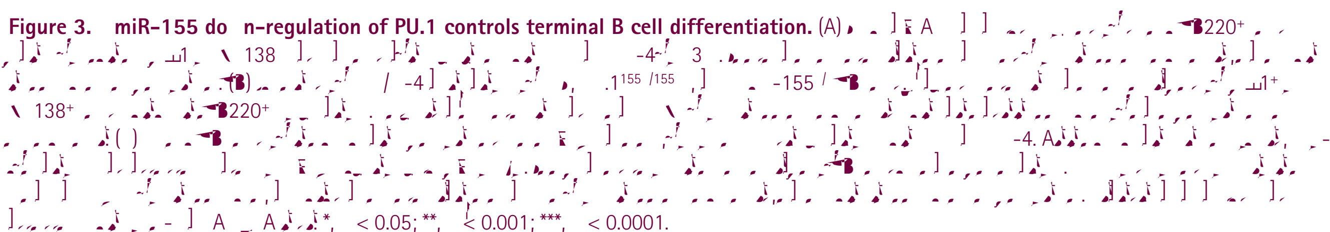

Figure 3. miR-155 do n-regulation of PU.1 controls terminal B cell differentiation. (A) ... B220+ ... 138 ... -4 ... 3 ... (B) ... -4 ... .1$^{155/155}$ ... -155$^{/}$ ... 1+ ... 138+ ... B220+ ... ( ) ... -4. A ... A ... *, < 0.05; **, < 0.001; ***, < 0.0001.

Pax5, and Blimp-1 is equivalent in naive B cells from WT, PU.1$^{155/155}$, and miR-155$^{/}$ mice. This result is consistent with the equivalent levels of Pax5 that we observed in developing

of PU.1 in PU.1$^{155/155}$ and miR-155$^{/}$ B cells, relative to WT, starting at day 1 of in vitro activation, followed by an increase in Pax5 and a decrease in Blimp-1 from day 3 (Fig. 4 B). Expression of Pax5 at day 3 suggests a slight delay in its upregulation in PU.1$^{155/155}$ B cells relative to miR-155$^{/}$ (Fig. 4 B). We do not know the basis for this di erence but it does not impact on Blimp-1 levels, which remain equivalent in

($P < 1.6 \times 10^{5}$; unpublished data). Moreover, using ingenuity pathways analysis, which combines gene ontology categories and curated literature, one of the top processes was "humoral immune responses: production of antibodies" ($P < 6.8 \times 10^{9}$; Fig. 6 C). This analysis led to the identification of Y30 PU.1 target genes with known links to hu

protein–protein interactions, critical processes in the context of T cell–dependent responses. Genes whose products are known to be important for sustaining interactions with T cells include Icosl, Pvrl1, Pd1d1l2, and Slamf1, although intrinsic roles in B cells for some of them remain to be assessed. Sema 7a, Sema 4a, and Cd300lf, the ligand Plexin-d1 (Plxnd1), and Fc-receptor genes (FcgRIIb, FcgRIV, and Fcrl5) are affected by PU.1 abundance. Proteins encoded by another group within the PU.1 targets are important in signal transduction downstream of some of the aforementioned receptors or the B cell receptor (Dap12, Rasgrp3, Gab2, and Card11). We also found that Ccr7 and Cxcr4, which have known roles in the migration of activated B cells, are regulated by PU.1. Furthermore, most of the genes we identified

Figure 6. PU.1 targets in activated B cells include genes involved in adhesion and B/T cellular interactions. (A) A [illegible]

di erent but complementary classes. Both groups are enriched in adhesion molecules and activation/di erentiation molecules. Collectively these results indicate that a large component of the transcriptome changes seen in miR-155 B cells relative to WT are mediated through a single target, namely PU.1.

DISCUSSION

It is well accepted that miRNAs have had a profound impact on the evolution of 3 UTRs and that a single miRNA can regulate the expression of hundreds of genes, although the level of repression imparted to a given target is generally low (Stark et al., 2005; Bartel, 2009). What is less evident is how the selective pressure for a single miRNA–target interaction is maintained across long evolutionary dis[illegible]

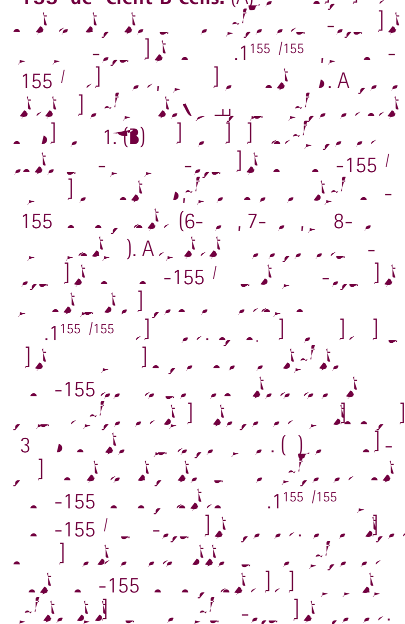

Figure 7. PU.1 explains a large fraction of changes in the transcriptome of miR-155 deficient B cells. (A)

though the miRNA has many additional targets, as indicated by the extensive overlap in the transcriptomes of PU.1$^{155/155}$ and miR-155$^{-/-}$-activated B cells. Despite the strong impact of PU.1 on miR-155 regulation of gene expression, we consistently observed that the fold-change in expression of DEGs relative to WT levels was higher in miR-155$^{-/-}$ than in PU.1$^{155/155}$ B cells. In other words, most of the DEGs in PU.1$^{155/155}$ B cells show intermediate expression relative to WT and miR-155$^{-/-}$. This is also manifested in the impaired Ig production in vivo, which is more severe in the miR-155$^{-/-}$ mice. At present we do not know the basis of this phenomenon but suggest that synergy with additional miR-155 targets may be occurring. It is likely that an answer to this will emerge from the analysis of in vivo activated B cells from PU.1$^{155/155}$ and miR-155$^{-/-}$ mice and this is an area that requires further investigation.

It is well recognized that effective transitions through developmental programs require strict control of the abundance of regulatory components. Posttranscriptional control of gene expression by miRNAs provides an effective mechanism to ensure timely transitions across developmental stages. In fact, dose-sensitive genes that regulate B cell activation in vivo, such as *Irf4*, *Bcl6*, *Aicda*, or *Prdm1*, are susceptible to miRNA regulation (de Yébenes et al., 2008; Teng et al., 2008; Malumbres

et al., 2009; West et al., 2009; Gururajan et al., 2010; Borchert et al., 2011; Chaudhuri et al., 2011; Lin et al., 2011; Huang et al., 2012). Although the signi cance of some of the aforementioned posttranscriptional regulation events remains to be explored in vivo, the interplay between transcription factors and miRNAs is emerging as a common theme in gene regulatory networks (Le et al., 2013). We show here that regulation of PU.1 abundance by miR-155 in activated B cells impacts on terminal B cell di erentiation in vivo and in vitro. The increased expression of PU.1, due to lack of miR-155-regulation, results in higher levels of Pax5 and lower levels of Blimp-1 concomitant with a reduction of plasma cells. Moreover, we were able to restore plasma cell di erentiation in miR-155$^{/}$ and PU.1$^{155/155}$ B cells by simply reducing Pax5 expression. In agreement with our results, sustained ectopic expression of Pax5 in murine splenocytes activated with LPS has been shown to inhibit plasma cell formation (Lin et al., 2002). In agreement with our results, Carotta et al. (this issue) have observed that overexpression or reduction of PU.1 expression in mature B cells a ected Pax-5 and Blimp1 expression, suggesting a role for PU.1 as a negative regulator of plasma cell di erentiation. Therefore, we propose that miR-155 regulates the initiation of the plasma cell di erentiation pathway through the inhibition of PU.1, which in turn regulates the expression of Pax5.

Genome-wide characterization of genes regulated transcriptionally by PU.1 shows that several of them encode membrane receptors with roles in cellular adhesion and intercellular communication. This functional pattern is consistent with previous reports mainly focused on myeloid cells (Turkistany and DeKoter, 2011), suggesting a broader role for PU.1 in regulates T cell function via binding of its ligand CD6 that appears to have inhibitory signaling function (Oliveira et al., 2012), whereas CD80 preferentially recruits the inhibitory molecule CTLA-4 to the T cell immunological synapse and may as a consequence inhibit T cell activation (Pentcheva-Hoang et al., 2004). Additional PU.1 targets include Sirpa and Adora, both of which regulate T cell activation and e ector function; however, their roles in B cells have yet to be studied. Overall, our results establish a novel negative regulatory function for PU.1 in activated B cells and identify a wide set of targets. In addition to Pax5 we have uncovered a set of genes with roles in cell adhesion and cellular communication that may regulate B–T cell interaction to mediate e ective immune responses. Our study also highlights the need to manipulate miRNA–target interactions in physiological settings to advance our understanding on miRNA biology. It is only in this way that we can formally establish cause–e ect relationships and distinguish epistasis between miRNAs and their targets.

MATERIALS AND METHODS

Mice. miR-155 mice, described previously (Rodriguez et al., 2007), were backcrossed six times to C57BL/6J. C57BL/6J mice were obtained from The Jackson Laboratory and were bred at the Babraham Institute. PU.1$^{155/155}$ mice were generated at the Babraham Institute (details below). All animal experimentation complied with UK Home O ce regulations and was approved by the local ethical review process at The Babraham Research Campus.

Generation of PU.1$^{155/155}$ mice and chimeras. The PU.1$^{155/155}$ targeting construct was derived from a previously described PU.1 knock-out targeting vector (Dakic et al., 2005). In brief, a fragment of 1.1-kb anking the miR-155–binding site in the 3 UTR of PU.1 was cleaved from the targeting vector using the restriction enzyme SalI. This was cloned into pBluescript SK in which site direct mutagenesis was performed using the Quick Change Multi Site kit (Agilent Technologies) with the following primers: 5 -GACCCCGCCGGCCATAGATGCATCCCGTCGCCCGGCCCGG-3 and 5 -CCGGGCCGGGCGACGGGATGCATCTATGGCCGGCGGGGTC-3 . The nucleotides underlined indicate those mutated. The mutation introduces an NsiI restriction site, which was used for genotyping purposes. Once the mutation had been sequence veri ed, the mutated fragment was cloned back into the targeting vector using the same SalI restriction site and checked for correct orientation by restriction pro le and sequencing. The linear targeting vector was transfected into C57BL/6 ES cells. Neomycin-resistant clones were screened by Southern hybridization and chimeras derived from blastocyst injection of these targeted clones were crossed to obtain germline transmission. FLPe mice were then crossed with the PU.1^{155GFP} mice for the removal of the IRES-GFP-Neomycin cassette and to obtain PU.1$^{155/+}$ mice. Further breeding produced the PU.1$^{155/155}$ homozygous mice used in this study. For the generation of mixed chimeras, 500-rads irradiated MMT mice received 5×10^6 of a mixture of 80% bone marrow cells of MMT origin and 20% WT, PU.1$^{155/155}$, or miR-155–de cient bone marrow cells. Reconstitution was assessed 6 wk later by measuring B and T cells from blood.

Luciferase assay. The *Sfpi1* 3 UTR was ampli ed from genomic DNA and inserted into the psiCheck-2 Renilla luciferase reporter plasmid (Promega, (Vigorito et al., 2007)). This construct was used to derive a miR-155 "seed" mutant plasmid with the Quik Change Multi Site Mutagenesis kit (Agilent Technologies). The mutagenic primers used were the same reported in the previous section to generate the knock-in mice. The correctness of all plasmids was con rmed by sequencing. Reporter assays were performed in

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RNAi gene silencing assay. The target sequence was introduced into the plasmid pMig empty retroviral vector via the BLOCK-iT polII miR RNAi vector kit (Invitrogen). The vectors were sequence con rmed before being introduced into the Plat-E retroviral packaging system with the X-treme GENE HP DNA transfection reagent (Roche). Retroviral supernatants were treated with PEG-iT virus precipitation solution (System Biosciences) for 12 h before centrifuged for 30 min at 500 g. All retroviral vectors expressed GFP, and an empty retroviral vector containing GFP only was used as the control.

Chromatin immunoprecipitation. Cultured B cells stimulated for 4 d with LPS (5 µg/ml) and IL-4 (10 ng/ml) were treated with 2% formaldehyde to cross-link the DNA and protein and incubated for 5 min at room temperature. This reaction was quenched by adding glycine to a nal concentration of 125 mM. Cells were then permeabilized in 5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% NP-40, and protease inhibitor cocktail (Roche) for 15 min at 4°C. Lysis bu er consisting of 1% SDS, 10 mM EDTA, and 50 mM Tris-HCl was applied to the permeabilized cells before fragmentation using a Diagenode Bioraptor UCD-200 sonicator.

Fragmented chromatin was diluted to 50 µg/ml in ChIP bu er, containing 0.01% SDS, 1.1% Triton-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, 167 mM NaCl, and protease inhibitors cocktail (Roche). 1 ml was used per immunoprecipitation reaction and 100 µl was used as input control. The chromatin was incubated with either 2.0 µg of rabbit anti-PU.1 (T-21 clone; Santa Cruz Biotechnology, Inc.) or the same amount of IgG isotype control overnight and precipitated using 50 µl protein A-coated magnetic beads (Invitrogen). Precipitates were then reverse cross-linked, mRNA and proteins were digested using RNase A and proteinase K by incubating at 37°C for 1 h and at 65°C overnight. After phenol/chloroform extraction, DNA was precipitated using isopropanol and resuspended in TE.

Library preparation for ChIP-Seq and RNA-Seq. ChIP-Seq libraries were constructed essentially following Illumina's standard ChIP-Seq library construction protocols. RNA-Seq libraries were constructed using the TruSeq sample preparation kit (Illumina) except that after the rst strand synthesis, the reaction mixture was cleaned up using the QIAQuick puri cation columns (QIAGEN) and the second strand synthesis was made using dUTP instead of dTTP. Just before the PCR ampli cation step, UNG (Ambion) was used to digest the second (opposite) strand containing uracil, to make it strand-speci c. Both ChIP and RNA libraries were run on the Bioanalyzer for quality control to check purity and size range.

Sequencing and read alignment. ChIP-Seq was performed on the Illumina Genome Analyser IIx using the 36-bp read length program. RNA-seq was performed on the Hi-Seq 2000 using the 75-bp read length program. The barcoded samples were then de-multiplexed and mapping was performed with the NCBIM37 (mm9) reference genome using Bowtie for ChIP-Seq and TopHat for RNA-Seq, respectively.

Peak calling and motif analysis for ChIP-seq. ChIP-Seq peaks were called using the default parameters on the MACS software version 1.3.6.1 (Zhang et al., 2008) and viewed using the SeqMonk program. For PU.1 motif discovery, the center 200 bp of called peaks were analyzed using the MEME suite program (Bailey et al., 2009) for alignment. For cis-regulatory motif analysis, the same sequences used in the MEME program were run in the RSAT pattern matching program using prede ned motifs (Heinz et al., 2010). For PU.1 motif discovery, the center 200 bp of called peaks were analyzed using the MEME suite program (Bailey et al., 2009) for alignment.

Transcriptome annotation and quanti cation. To determine whether a given gene is de ned as "expressed," an initial quantitation was made by counting the number of RPKM, where the normal distribution was viewed and an expression cut-o of RPKM=1 was chosen. To identify signi cantly changing genes, RPM values were quantitated. Di erential expression was called by selecting transcripts, which changed with a signi cance of $P < 0.05$ after Benjamini and Hochberg correction using a null model constructed

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