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SLP-65 regulates immunoglobulin light chain gene recombination through the PI(3)K-PKB-Foxo pathway

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Although the essential role of the adaptor protein SLP-65 in pre-B cell differentiation is established, the molecular mechanism underlying its function is poorly understood. In this study, we uncover a link between SLP-65–dependent signaling and the phosphoinositide-3-OH kinase (PI(3)K)–protein kinase B (PKB)–Foxo pathway. We show that the forkhead box transcription factor Foxo3a promotes light chain rearrangement in pre-B cells. Our data suggest that PKB suppresses light chain recombination by phosphorylating Foxo proteins, whereas reconstitution of SLP-65 function counteracts PKB activation and promotes Foxo3a and Foxo1 activity in pre-B cells. Together, these data illuminate a molecular function of SLP-65 and identify a key role for Foxo proteins in the regulation of light chain recombination, receptor editing and B cell selection.

In humans and mice, mature B lymphocytes are generated in a precisely regulated developmental process that includes the assembly of B cell receptor variable region exons from germline variable (V), diversity (D) and joining (J) gene segments for the immunoglobulin heavy chain and V and J segments for the immunoglobulin light chain[1]. V(D)J recombination is mediated by the lymphocyte-specific RAG-1 and RAG-2 proteins, which introduce double-strand breaks between gene segments and flanking recombination signal sequences (RSS)[2]. The cellular DNA repair machinery then processes and rejoins the breaks[3].

First initiated on the heavy chain locus, successful in-frame V(D)J-rearrangements in pro-B cells generate an immunoglobulin heavy chain that forms a pre-B cell receptor (pre-BCR) when expressed on the cell surface together with the surrogate light chain components VpreB and λ5 and the signaling subunits immunoglobulin-α and immunoglobulin-β (ref. 4). This pre-B cell stage represents an important checkpoint in B cell development, as signals from the pre-BCR provide instant feedback about the functionality of the recombined heavy chain, enabling only pre-B cells with a signaling-competent receptor to progress onward.

A key component in this quality control checkpoint is the adaptor protein SLP-65, which mediates signaling downstream of the pre-BCR and the BCR[4]. When phosphorylated by the tyrosine kinase Syk, SLP-65 promotes the formation of signaling complexes by recruiting molecules containing SH2 domains such as the kinase Btk and phospholipase C (PLC)-γ2, which connect receptor activation to downstream effectors. The crucial role of SLP-65 in B cell development is demonstrated by the phenotype of mice in which the gene encoding SLP-65 (*Blnk*) is deleted. In SLP-65–deficient mice, B cell development is partially blocked at the pre-B cell stage, resulting in a reduction of mature B cells in the periphery. SLP-65–deficient cells express large amounts of the pre-BCR on their surface and proliferate at a higher rate than wild-type cells. Notably, about 7% of SLP-65–deficient mice develop leukemia[5]. It is therefore assumed that SLP-65–independent pre-BCR signals induce proliferation, whereas SLP-65–dependent signals promote differentiation. However, the molecular mechanism through which SLP-65 enhances differentiation rather than proliferation remains unclear.

Pre-BCR signals initiate rearrangement of light chain gene segments, which leads to the expression of the B cell receptor (BCR) on immature B cells[4]. The inherent variability in the rearrangement of heavy chain and light chain gene segments necessitates selection processes to handle lymphocytes that either lack a functional BCR or express a BCR that recognizes self antigen[6]. In addition to clonal deletion and anergy induction, central B cell tolerance is established through receptor editing, wherein autoreactive BCR signaling promotes further rearrangements of light chain gene segments[7]. This process sometimes allows replacement of the autoreactive receptor with a non-autoreactive receptor, and thereby eliminates the autoreactivity of the cell without eliminating the cell itself.

The exact molecular signaling pathways involved in receptor editing have yet to be defined. It is evident that antigen-mediated BCR signaling in immature B cells promotes editing, whereas further rearrangements are suppressed in non-autoreactive cells. This suggests that tonic BCR signals actively counteract secondary recombination. Indeed, inhibiting tyrosine kinases in a model system of immature B cells promotes RAG-2 reexpression and new light chain rearrangements[8]. In addition, Syk-deficient non-autoreactive cells undergo

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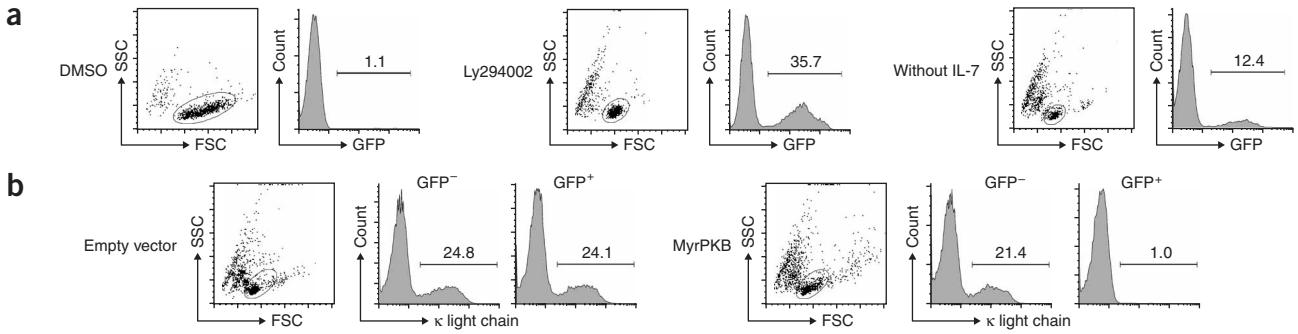

Figure 1 Signaling through PI(3)K and PKB regulates light chain recombination in pre-B cells. (**a**) GFP reporter recombination in SLP-65–deficient pre-B cells in the absence or presence of a PI(3)K inhibitor. Cells expressing the recombination reporter were treated with the PI(3)K inhibitor Ly294002 in the presence of IL-7 or DMSO (negative control) or in the absence of IL-7 (positive control). After 60 h, GFP expression was analyzed by flow cytometry. Dot plots display size of treated cells on the FSC axis. Numbers in histograms indicate the percentages of GFP$^+$ cells. (**b**) Differentiation of SLP-65–deficient pre-B cells expressing a constitutively active form of PKB. Cells transduced with the empty vector as a control (IRES-GFP) or with the vector encoding constitutively active myristoylated PKB (myrPKB-IRES-GFP) were cultured without IL-7 for 2 d and analyzed by flow cytometry. κ light chain surface expression was compared in the GFP$^-$ and the GFP$^+$ populations within each sample. The percentages of cells in the respective regions are indicated. Data are representative of at least six independent experiments (**a**,**b**).

receptor editing, suggesting that Syk transmits signals that normally suppress recombination[9]. However, SLP-65 and PLC-γ2, situated downstream of Syk, seem important for efficient receptor editing, as secondary light chain rearrangements are impaired when one of these molecules is absent[10,11].

Also involved in tonic BCR signaling is the PI(3)K pathway[8,12,13]. When activated, PI(3)K phosphorylates its substrate phosphatidylinositol-4,5-bisphosphate, thereby generating the second messenger phosphatidylinositol-3,4,5-trisphosphate. Signaling proteins such as the serine-threonine kinase PKB (also called Akt) and its activating kinase PDK1 contain a pleckstrin homology domain, which binds to phosphatidylinositol-3,4,5-trisphosphate, thereby mediating recruitment of the respective protein to the cellular membrane. PKB is important in mediating cellular proliferation and survival induced by PI(3)K[14].

Principal targets of PI(3)K-PKB signaling are Foxo transcription factors, a subgroup of the FOX family that share the forkhead box, a highly conserved DNA binding domain[15]. Foxo transcription factors are the mammalian homologs of the *Caenorhabditis elegans* gene *daf-16* and comprise Foxo1, Foxo3a, Foxo4 and Foxo6, with Foxo3a being the dominant isoform expressed in lymphocytes[15]. Knockout studies have established a role for Foxo3a in mediating hematopoietic stem cell resistance to oxidative stress, lymphocyte homeostasis and prevention of autoimmunity[16,17], which is in agreement with data showing a decreased activity of Foxo3a in autoimmune-prone mouse strains[16]. In various cellular systems, Foxo transcription factors regulate diverse genes, such as those encoding cell cycle and survival machinery[15]. Recent data have validated a role for Foxo transcription factors as tumor suppressors in mice[18]. The activity of Foxo transcription factors is mainly regulated by PKB-mediated phosphorylation on conserved residues, which promotes their nuclear export and proteasomal degradation[19–21].

In this study, we analyzed the molecular pathways that are involved in the regulation of light chain recombination in pre-B and immature B cells. We found that primary and secondary light chain recombination was tightly controlled by tonic receptor signaling through PI(3)K and PKB. As one target of this pathway, we identified Foxo3a, which promotes light chain rearrangements when uncoupled from tonic signals. SLP-65 counteracted PKB activity and thereby promoted activation of Foxo3a and Foxo1.

RESULTS

PI(3)K and PKB activity inhibit differentiation

To identify pathways that are involved in regulation of pre-B cell differentiation, we used a reporter system that enabled us to monitor the activity of the recombination machinery in living cells (**Supplementary Fig. 1** online). This system is based on a construct in which an inversely orientated cDNA encoding GFP is flanked by two RSS. Under conditions that allow recombination, RAG-1 and RAG-2 invert the GFP cDNA, resulting in GFP expression from the retroviral promoter. Previous data show that this system is an appropriate tool to screen for recombination events in pre-B cells[22].

Mice lacking the PI(3)K regulatory subunit p85α show severe defects in B cell development with a block at the pro-B to pre-B cell transition and reduced numbers of mature B cells[23], raising the possibility that PI(3)K signaling is involved in pre-B cell differentiation. To investigate this question, we treated a SLP-65–deficient pre-B cell line selected for expression of the reporter construct with the PI(3)K inhibitor Ly294002 in the presence of IL-7 and analyzed DNA recombination events as measured by expression of GFP after 60 h (**Fig. 1a**). As controls, we used cells treated with DMSO, as well as cells subjected to IL-7 withdrawal—a treatment established to induce *Igk* recombination in pre-B cells in culture. Cells cultured in presence of IL-7 retained their normal size, as measured by forward scatter flow cytometry (FSC), and did not recombine the reporter construct (**Fig. 1a**). In contrast, withdrawal of IL-7 resulted in an increase of GFP$^+$ cells and a clear reduction in cell size, which in this context reflects a halt in cellular proliferation. Notably, cells cultured in presence of the PI(3)K inhibitor Ly294002 resembled the cells cultured without IL-7 both in reduced cell size and in recombination of the reporter substrate. These data suggest that signaling through PI(3)K promotes proliferation and suppresses pre-B cell differentiation under normal conditions.

Given that PKB is a principal target of PI(3)K signaling, we determined whether activated PKB suppresses differentiation even in the absence of IL-7. We transduced SLP-65–deficient pre-B cells with an empty vector (IRES-GFP) as a control or with a bicistronic construct encoding GFP as a marker and a constitutively active myristoylated form of PKB[24]. After culture without IL-7 for 2 d to promote light chain recombination, pre-B cells expressing IRES-GFP but not those expressing the constitutively active PKB differentiated

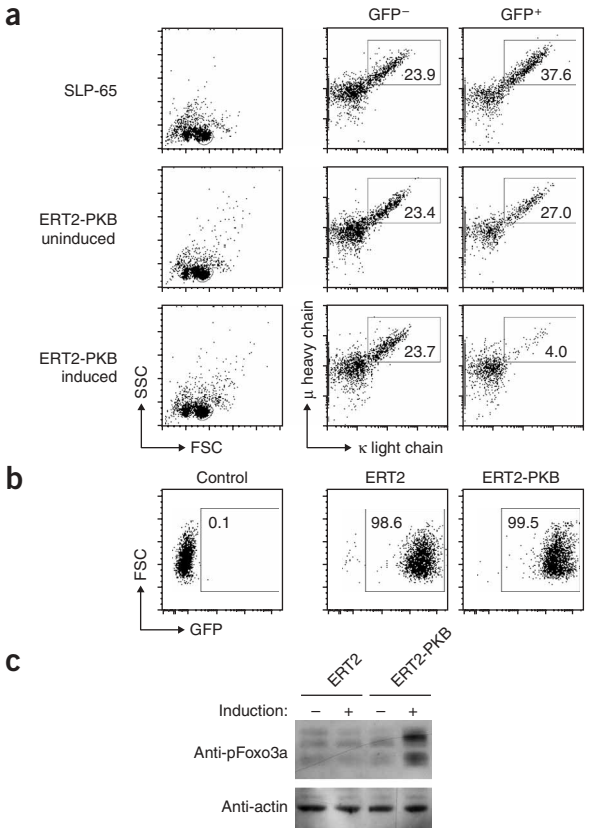

Figure 2 PKB targets Foxo3a in pre-B cells. (**a**) Differentiation of pre-B cells expressing an inducible form of PKB. Pre-B cells transduced with a construct encoding either SLP-65 as a positive control or a 4-OHT–inducible form of PKB (ERT2-PKB) were cultured without IL-7 and treated with 4-OHT (induced) or the solvent ethanol (uninduced). After 60 h, cell size and κ light chain surface expression were analyzed by flow cytometry. The numbers indicate the percentages of cells in the respective regions. (**b**) Flow cytometry of pre-B cells used for the Foxo3a phosphorylation assay in **c**. Pre-B cells lacking SLP-65, RAG-2 and λ5 (TKO) were transduced with vectors encoding the inducible PKB (ERT2-PKB-IRES-GFP) or only the ERT2 domain (ERT2-IRES-GFP). Boxes, sorted GFP⁺ cells. Untransduced cells are shown as a control. (**c**) Phosphorylation of Foxo3a upon activation of PKB. Sorted cells shown in **b** were treated with 4-OHT (+) or the solvent ethanol (–) for 4 h, lysed and subjected to SDS-PAGE. Phosphorylated Foxo3a (pFoxo3a) was detected by immunoblot. Actin, loading control. Data are representative of at least five independent experiments (**a**,**c**).

into BCR⁺ cells as measured by κ light chain surface expression (**Fig. 1b**). Together, our data indicate that signaling through PI(3)K and PKB counteracts differentiation in pre-B cells.

PKB phosphorylates Foxo3a in pre-B cells

To further investigate PKB function in pre-B cells, we generated a tamoxifen-inducible PKB construct (ERT2-PKB) by fusing PKB to the mutated estrogen receptor ligand-binding domain ERT2 (ref. 25). Such PKB fusion proteins allow the induction of PKB function in a temporally regulated manner[26]. To test the effect of this construct in our system, we transduced a SLP-65–deficient pre-B cell line with retroviral vectors encoding either SLP-65 as a positive control or ERT2-PKB; both retroviruses contained IRES-GFP cassettes to mark infected cells. Transduced cells were cultured without IL-7 and treated with the inducer 4-hydroxytamoxifen (4-OHT) or its solvent ethanol as indicated. Flow cytometry analysis after 60 h showed that in ERT2-PKB-transduced cells, administration of 4-OHT resulted in a decreased proportion of cells expressing surface κ light chain (**Fig. 2a**). In contrast, retroviral expression of SLP-65 resulted in an increase in the proportion of κ⁺ cells.

As a putative target of PKB, we investigated Foxo3a because recent data show an involvement of Foxo3a in differentiation of BCR-Abl–transformed pre-B cells[27]. To test whether Foxo3a was a target of PKB in our pre-B cells, we transduced triple knockout (TKO) cells lacking RAG-2, SLP-65 and λ5 with retroviral vectors encoding either the inducible form of PKB (ERT2-PKB-IRES-GFP) or only the ERT2 domain (ERT2-IRES-GFP) as a control, and sorted the GFP⁺ cells (**Fig. 2b**). Sorted TKO cells were treated with 4-OHT or ethanol for 4 h and total cellular lysates were immunoblotted. 4-OHT treatment induced phosphorylation of Foxo3a in cells expressing ERT2-PKB but not in those expressing ERT2 alone (**Fig. 2c**), demonstrating that Foxo3a is a substrate for PKB in pre-B cells.

Foxo3a induces light chain expression in pre-B cells

The identification of Foxo3a as a target of PKB in pre-B cells raised the question of whether the light chain recombination and inhibition of differentiation induced by constant PI(3)K-PKB signaling was directly mediated by constant degradation of Foxo3a. To investigate this possibility, we transduced a SLP-65-deficient pre-B cell line with an empty vector as a control or a vector encoding a Foxo3a mutant in which the residues normally phosphorylated by PKB had been

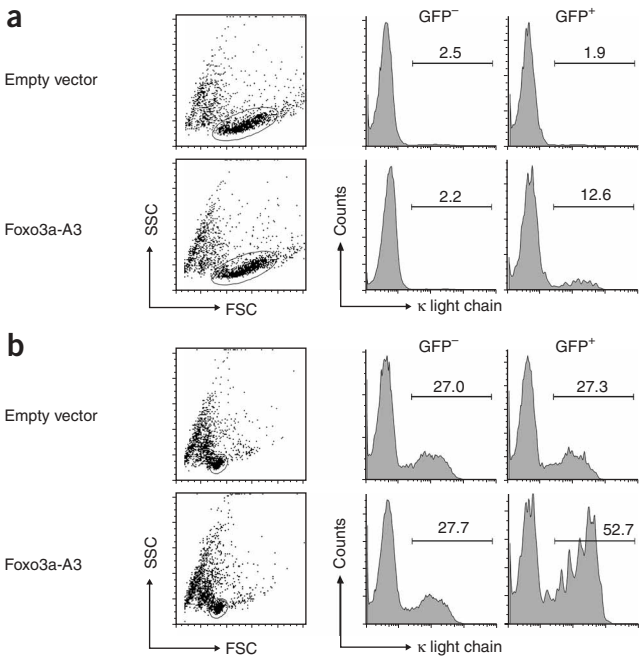

Figure 3 Foxo3a-A3 promotes differentiation of pre-B cells. (**a**,**b**) Differentiation of pre-B cells expressing a constitutively active form of Foxo3a. SLP-65–deficient pre-B cells were transduced with a vector encoding a constitutively active form of Foxo3a (Foxo3a-A3–IRES–GFP) or the empty vector as a control and cultured either with (**a**) or without (**b**) IL-7 for 48 h. Differentiation of pre-B cells, indicated by cell size (left) and surface κ light chain expression (right) was measured by flow cytometry in GFP⁻ and the GFP⁺ population within each sample. The numbers indicate the percentages of cells in the respective regions. Data are representative of four independent experiments (**a**,**b**).

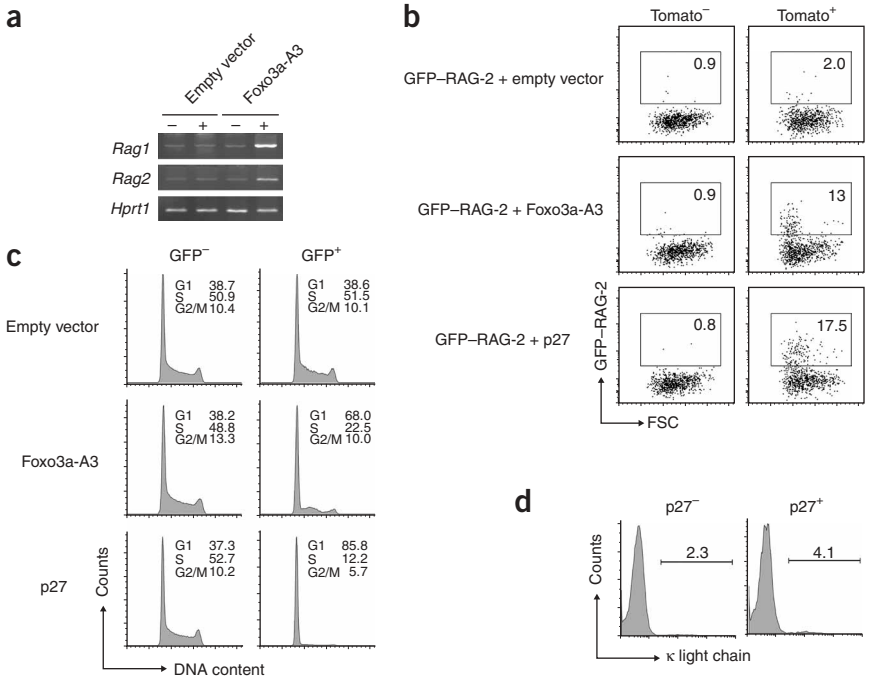

Figure 4 Foxo3a-A3 prolongs the G1 phase of the cell cycle and stabilizes RAG-2. (**a**) Foxo3a-mediated transcription of *Rag1* and *Rag2*. SLP-65–deficient pre-B cells were transduced with a vector encoding Foxo3a-A3 or an empty control vector. After 36 h, cells were sorted into untransduced (–) and transduced (+) populations. cDNA was generated from total RNA and PCR was performed using gene-specific primers. *Hprt1*, loading control. (**b**) Stabilization of a GFP–RAG-2 fusion protein by Foxo3a-A3 in pre-B cells. SLP-65–deficient pre-B cells were transduced with a vector encoding GFP–RAG-2 and either an empty vector (IRES-tomato), a vector encoding Foxo3a-A3 (Foxo3a-A3–IRES–tomato) or, as a positive control, a vector encoding p27 (p27-IRES-tomato). After 48 h, expression of GFP–RAG-2 and cell size was compared between the tomato⁻ and the tomato⁺ population within each sample by flow cytometry. The numbers indicate the percentages of GFP⁺ cells in the marked regions. (**c**) Cell cycle analysis of pre-B cells expressing Foxo3a-A3. SLP-65–deficient pre-B cells were transduced with either an empty control vector (IRES-GFP), a vector encoding Foxo3a-A3 (Foxo3a-A3–IRES–GFP) or a vector encoding p27 (p27-IRES-GFP). Cells were fixed after 48 h, stained for DNA content and analyzed by flow cytometry. The numbers indicate the percentages of cells in designated cell cycle phases. (**d**) κ light chain surface expression in pre-B cells expressing p27. SLP-65–deficient pre-B cells were transduced with a vector encoding p27, cultured with IL-7 and κ light chain surface expression was measured by flow cytometry after 48 h. The numbers indicate the percentages of cells in the respective regions. Data are representative of three (**a**,**b**), two (**c**) or four (**d**) independent experiments.

mutated to alanine (Foxo3a-A3). Transduced cells were cultured either with or without IL-7, and κ light chain expression was measured by flow cytometry after 48 h (**Fig. 3a**,**b**). In the presence of IL-7, cells transduced with the empty vector did not express κ light chain efficiently; however, expression of the Foxo3a-A3 mutant—regardless of the presence or absence of IL-7—induced an increase in the percentage of κ^+ cells, indicating that Foxo3a can induce κ light chain expression even in the presence of IL-7 (**Fig. 3a**,**b**).

Under normal conditions, the activity of the recombination machinery that mediates *Ig* gene recombination is limited. RAG-2 protein accumulates during the G1 phase of the cell cycle and is subsequently degraded upon entry into the S phase[28], whereas RAG-1 might be regulated mainly by transcription. RT-PCR experiments on RNA from Foxo3a-A3–expressing cells showed a clear induction of *Rag1* and *Rag2* mRNA relative to expression in control cells (**Fig. 4a**). To analyze RAG-2 stabilization, we transduced a SLP-65–deficient pre-B cell line with a construct encoding a GFP–RAG-2 fusion protein, together with either an empty control vector encoding the red fluorescent protein tdTomato (IRES-tomato), a vector encoding Foxo3a-A3 (Foxo3a-A3–IRES–tomato) or a vector encoding p27 (p27-IRES-tomato)[29,30]. After 48 h in culture with IL-7, we analyzed the cells by flow cytometry (**Fig. 4b**). In agreement with published data, pre-B cells transduced with the GFP–RAG-2 vector did not express detectable GFP in the presence of IL-7 (refs. 29,30). However, expression of the cell cycle inhibitor p27 or withdrawal of IL-7 resulted in stabilization of GFP–RAG-2 and detection of GFP^+ cells (**Fig. 4b** and not shown). Like expression of p27, expression of Foxo3a-A3 in SLP-65–deficient pre-B cells resulted in a marked increase in the percentage of GFP^+ cells and an accompanying reduction in cell size, as measured by FSC. These observations suggest that Foxo3a counteracts cell cycle progression, thereby leading to RAG-2 protein stabilization. To confirm this hypothesis, we determined by flow cytometry the DNA content of the SLP-65–deficient pre-B cell line transduced with either an empty control vector (IRES-GFP) or vectors encoding Foxo3a-A3 (Foxo3a-A3–IRES–GFP) or p27 (p27-IRES-GFP). Expression of either Foxo3a-A3 or p27 resulted in an increase in the proportion of cells in the G1 phase and a decrease in the percentage of cycling cells (**Fig. 4c**). However, in contrast to Foxo3a-A3 (**Fig. 3a**), p27 was unable to efficiently activate light chain surface expression (**Fig. 4d**) despite the very efficient p27-induced cell cycle arrest and RAG-2 stabilization. This finding suggests that blocking the cell cycle by itself is not sufficient to activate light chain expression and that specific Foxo-dependent functions are required.

Foxo3a-A3 counteracts PKB in pre-B cells

Our data indicated that signaling through PI(3)K and PKB phosphorylates Foxo3a and inhibits light chain recombination and differentiation, whereas a stabilized form of Foxo3a induces light chain expression and differentiation. This suggested that the Foxo3a-A3 mutant may counteract the block in pre-B cell differentiation induced by constitutively active PKB. To address this point, we transfected our SLP-65–deficient pre-B cell line with a vector encoding ERT2-PKB, together with either an empty control vector (IRES-tomato; **Fig. 5a**) or a vector encoding Foxo3a-A3 (Foxo3a-A3–IRES–tomato; **Fig. 5b**). To induce differentiation, the transduced cells were cultured without IL-7 in the presence of 4-OHT or the solvent ethanol, and κ light chain expression was measured by flow cytometry after 48 h. Compared to cells transduced with the empty control vector, a higher proportion of cells transduced with Foxo3a-A3–IRES–tomato expressed surface κ light chain after ethanol treatment (**Fig. 5a**,**b**). ERT2-PKB induction more effectively inhibited κ light chain expression in control than in Foxo3a-expressing populations. These results demonstrate that a Foxo3a mutant that is uncoupled from the regulation by PKB is sufficient to induce κ light chain expression even in the presence of

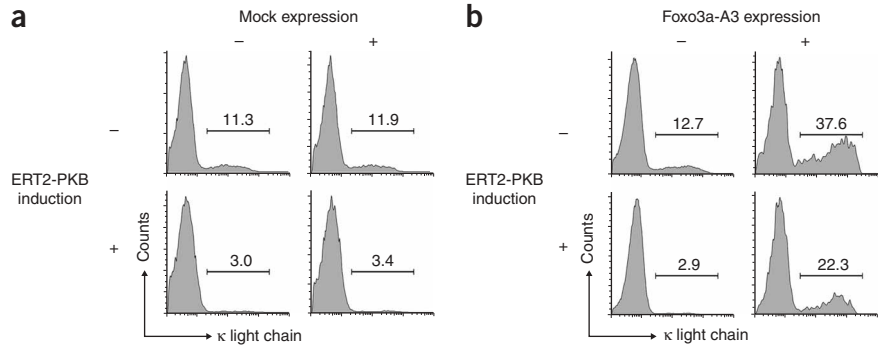

Figure 5 Foxo3a-A3 counteracts the block in κ light chain expression in pre-B cells mediated by constitutively active PKB. (**a**,**b**). κ light chain expression on pre-B cells expressing an inducible form of PKB together with active Foxo3a-A3. SLP-65–deficient pre-B cells were transduced with a vector encoding ERT2-PKB (ERT2-PKB-IRES-GFP) and either an empty control vector (IRES-tomato, mock, **a**) or a vector encoding Foxo3a-A3 (Foxo3a-A3–IRES–tomato, **b**), and were cultured without IL-7 together with 4-OHT (+) or the solvent ethanol (–). κ light chain expression was analyzed by flow cytometry 48 h later. The histograms depict cells gated for expression of ERT2-PKB. The numbers indicate the percentages of cells in the marked regions. Data are from one of three independent experiments with similar results (**a**,**b**).

constitutively active PKB. In addition, these findings suggest that inhibition of Foxo proteins is an important event required for PKB-mediated suppression of light chain recombination.

To further address the role of Foxo3a in the regulation of light chain expression and pre-B cell differentiation, we generated Foxo3a-deficient pre-B cells by crossing mice having a *loxP*-flanked *Foxo3* gene[31] to mice expressing the Cre recombinase specifically in B cells under the *Cd79a* promoter[32]. After 4 d of *in vitro* culture in the presence of IL-7, Foxo3a-deficient pre-B cells and Foxo3a-sufficient control cells, each negative for immunoglobulin M (IgM), were sorted (**Supplementary Fig. 2a** online) and cultured for another 2 d in the absence of IL-7. We noted a decreased proportion of IgM^+ cells among the Foxo3a-deficient pre-B cells as compared with control cells (**Fig. 6**). Inactivation of the *Foxo3* gene by *Cd79a-Cre* and nontoxicity of Cre were confirmed by immunoblotting and flow cytometry (**Supplementary Fig. 2b**,**c**). In addition, mice lacking Foxo3a in B cells showed a reduction of mature recirculating B cells in the bone marrow, suggesting that Foxo3a is required for normal B cell development (**Supplementary Fig. 2d**). On the other hand, the obvious partial differentiation capacity of the Foxo3a-deficient pre-B cells indicates that other Foxo family members may partially substitute Foxo3a in *Ig* gene recombination and B cell differentiation.

Our data implied that Foxo3a induces differentiation of pre-B cells and blocks progression through the cell cycle, raising the question of how Foxo3a activity is regulated during B cell development. To assess the effects of receptor signaling on PKB and Foxo3a phosphorylation, we reconstituted TKO pre-B cells with vectors encoding the μ heavy chain and either λ5 or λ light chain to generate a pre-BCR or BCR, respectively (**Fig. 7a**). Parental, receptor-negative TKO cells and cells sorted for expression of the pre-BCR and the BCR, respectively, were lysed and analyzed for phosphorylation of PKB and Foxo3a by immunoblot. PKB activity, as measured by PKB phosphorylation, was reduced in receptor-negative TKO cells, and Foxo3a was only weakly phosphorylated (**Fig. 7b**). In contrast, expression of a pre-BCR strongly induced phosphorylation of both PKB and Foxo3a (**Fig. 7b**), indicating that autonomous receptor signaling activates PI(3)K and downstream components. We obtained comparable results in a different cell line also (**Supplementary Fig. 3** online). Notably, TKO cells expressing the BCR also showed PKB and Foxo3a phosphorylation (**Fig. 7b**).

As Foxo3a was constitutively phosphorylated in pre-BCR^+ cells lacking SLP-65, we tested whether SLP-65–dependent pre-BCR signaling interfered with PI(3)K and PKB activity, as would be expected from the role of SLP-65 in promoting differentiation[5]. To test this hypothesis, we treated pre-BCR^+ TKO cells reconstituted with ERT2-SLP-65 with 4-OHT or the solvent ethanol for 4 and 8 h before lysis and immunoblot analysis of PKB and Foxo3a phosphorylation. Compared to ethanol, 4-OHT treatment resulted in a marked decrease in PKB and Foxo3a phosphorylation (**Fig. 7c**). This observation suggests that pre-BCR signaling mediated by SLP-65 counteracts PKB and activates Foxo3a.

The residual differentiation capacity of the Foxo3a-deficient pre-B cells suggested that other Foxo proteins might have redundant functions in B cells, as described previously for many cell types[17,18]. Therefore, we tested whether SLP-65 and PKB also influence Foxo1, which is expressed ubiquitously[15]. Like Foxo3a, Foxo1 was phosphorylated by receptor-induced PKB activation, and this phosphorylation was inhibited by SLP-65–induced inactivation of PKB (**Fig. 7d**,**e**). Together, these data show that SLP-65 is required for efficient activation of Foxo transcription factors in B cells.

SLP-65 regulates Foxo3a in BCR^+ cells

By analogy to the pre-BCR, we also investigated the role of SLP-65 in down-modulating PKB phosphorylation in BCR^+ cells. As a major difference between the BCR and the pre-BCR is the autonomous signaling of the latter, BCR^+ TKO cells reconstituted with ERT2-SLP-65 were stimulated with their corresponding antigen, nitro-iodo-phenol (NIP)-BSA, in addition to the treatment with 4-OHT or the solvent ethanol. Cross-linking the BCR by NIP-BSA or activation of ERT2-SLP-65 by administration of 4-OHT alone resulted in only a slight decrease in PKB phosphorylation compared to that in unstimulated control cells (**Fig. 8a**). However, a combination of receptor cross-linking and SLP-65 induction markedly reduced PKB activity, which in turn was also reflected by a drop in Foxo3a phosphorylation (**Fig. 8a**).

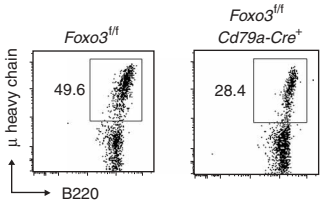

Figure 6 Foxo3a-deficient pre-B cells show impaired differentiation into BCR^+ cells *in vitro*. Differentiation of bone marrow-derived pre-B cells from $Foxo3^{f/f}Cd79a\text{-}Cre^+$ and $Foxo3^{f/f}$ mice. Bone marrow cells were cultured in the presence of IL-7 for 4 d, and $B220^+IgM^-$ pro-/pre-B cell populations were sorted and incubated without IL-7 for 2 d before flow cytometry analysis. The numbers indicate the percentages of BCR^+ immature B cells ($B220^+IgM^+$) in the respective cultures. Data are from one of four independent experiments with similar results.

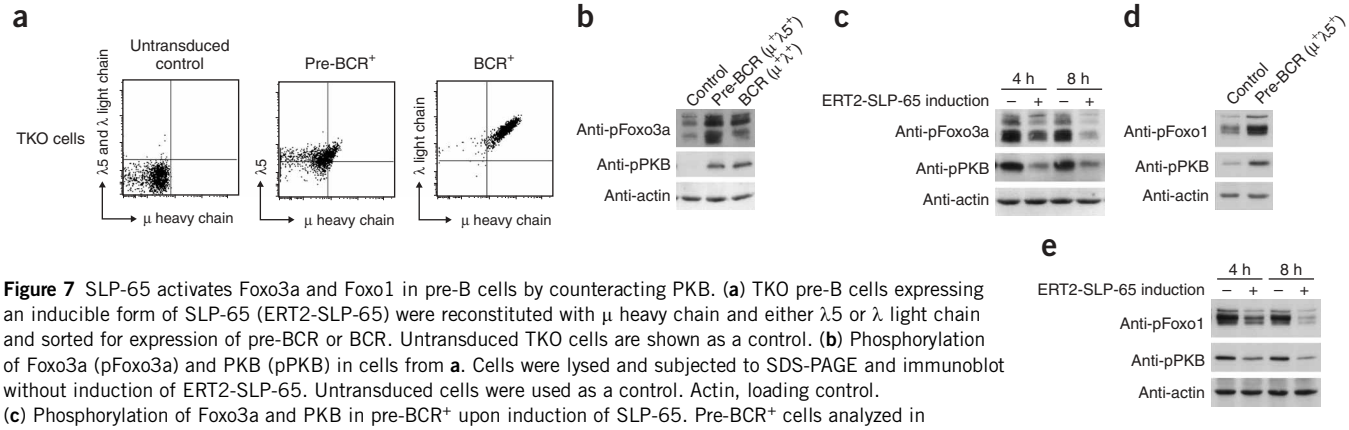

Figure 7 SLP-65 activates Foxo3a and Foxo1 in pre-B cells by counteracting PKB. (**a**) TKO pre-B cells expressing an inducible form of SLP-65 (ERT2-SLP-65) were reconstituted with μ heavy chain and either λ5 or λ light chain and sorted for expression of pre-BCR or BCR. Untransduced TKO cells are shown as a control. (**b**) Phosphorylation of Foxo3a (pFoxo3a) and PKB (pPKB) in cells from **a**. Cells were lysed and subjected to SDS-PAGE and immunoblot without induction of ERT2-SLP-65. Untransduced cells were used as a control. Actin, loading control. (**c**) Phosphorylation of Foxo3a and PKB in pre-BCR$^+$ upon induction of SLP-65. Pre-BCR$^+$ cells analyzed in **a** were treated with 4-OHT (+) or the solvent ethanol (–) for 4 and 8 h before lysis, SDS-PAGE and immunoblot. (**d**,**e**) Analogous regulation of Foxo1 by SLP-65–independent and SLP-65–dependent pre-BCR signals. Cells shown in **a** were treated as in **b** and **c**, with immunoblot for Foxo1 instead of Foxo3a. Data are from one of four (**b**), six (**c**) or two (**d**,**e**) independent experiments with similar results.

This result suggests that strong signals from the BCR downmodulate PKB activity in the presence of SLP-65, raising the question whether the PI(3)K-PKB pathway and Foxo proteins also play a role in regulating secondary light chain recombination in BCR$^+$ cells. As this point cannot be addressed in the TKO cells that lack RAG-2 and are therefore unable to recombine light chain gene segments, we used a RAG-2–expressing, SLP-65–deficient cell line that expresses an endogenous BCR owing to spontaneous differentiation *in vitro*. This cell line was transduced with the recombination reporter and treated with the PI(3)K inhibitor Ly294002 or the solvent DMSO. After 36 h, we analyzed cell size, κ light chain surface expression and expression of GFP as a marker for recombination by flow cytometry. Consistent with the data in pre-B cells, inhibition of PI(3)K resulted in an increase in the proportion of GFP$^+$ cells as compared to that in cells treated only with DMSO, and it resulted in a marked reduction in cell size (**Fig. 8b**). Moreover, a substantial fraction of GFP$^+$ cells showed decreased κ light chain surface expression, whereas a smaller fraction showed increased κ light chain surface expression. Taken together, these data indicate that the PI(3)K-PKB pathway is not only important for the suppression of recombination in pre-B cells, but is also key in the regulation of secondary light chain rearrangements in BCR$^+$ cells.

Given that constant signaling through PI(3)K inhibits the recombination machinery in BCR$^+$ cells, we tested whether expression of the

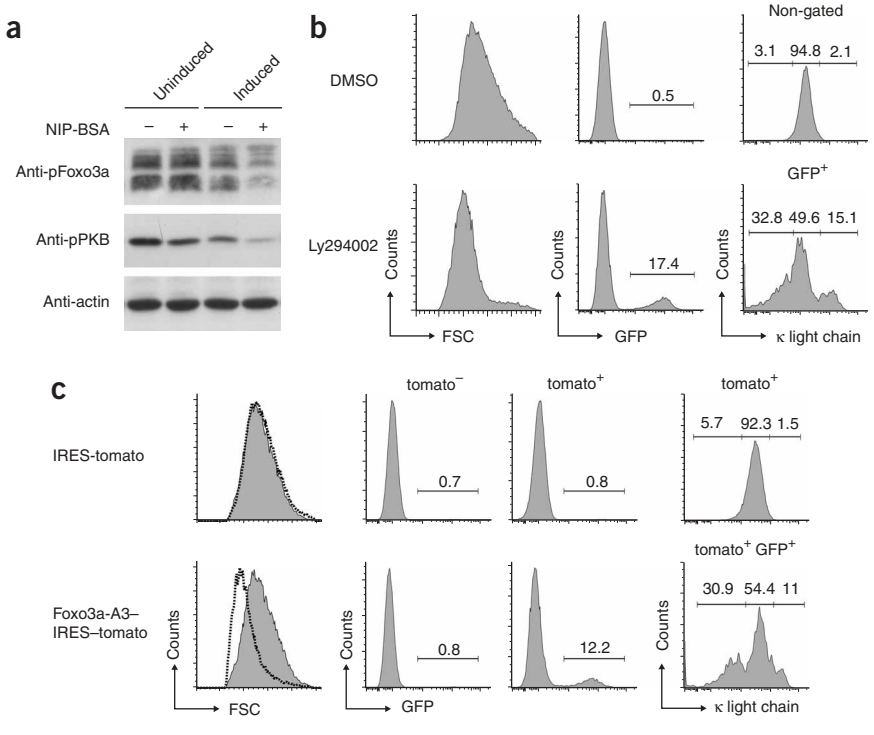

Figure 8 SLP-65 signaling regulates the PI(3)K-PKB-Foxo3a pathway in BCR$^+$ cells. (**a**) BCR-induced SLP-65–dependent Foxo3 activation. TKO BCR$^+$ cells reconstituted with ERT2-SLP-65 were treated with NIP-BSA, 4-OHT or both for 8 h before lysis, SDS-PAGE and immunoblot. Cells treated with the solvent ethanol (uninduced) were used as a control. Immunoblots display phosphorylation of Foxo3a (pFoxo3a) and PKB (pPKB). Actin, loading control. (**b**) Recombination of the GFP reporter substrate upon inhibition of PI(3)K. SLP-65–deficient BCR$^+$RAG-2$^+$ cells were transduced and selected for expression of the recombination reporter as shown in **Figure 1a**. After 36 h of treatment with the PI(3)K inhibitor Ly294002 or the solvent DMSO, cell size, GFP and κ light chain surface expression was measured by flow cytometry in the total population (for DMSO-treated cells) or in gated GFP$^+$ cells (for Ly294002-treated cells). The numbers indicate the percentages of cells in the respective regions. (**c**) Foxo3-A3–induced recombination in BCR$^+$ cells. SLP-65–deficient BCR$^+$ cells expressing the recombination reporter were transduced with a vector encoding Foxo3a-A3 (Foxo3a-A3–IRES–tomato) or the empty vector as a control (IRES-tomato) and analyzed by flow cytometry after 48 h. Histogram overlays show tomato$^-$ (filled) and tomato$^+$ (dotted line) populations. Histograms compare GFP expression in the tomato$^-$ and the tomato$^+$ populations within each sample. κ light chain expression was analyzed in tomato$^+$ cells transduced with the empty vector and tomato$^+$GFP$^+$ cells transduced with the vector encoding Foxo3a-A3. The numbers indicate the percentages of cells in the respective regions. Data are representative for four (**a**) or five (**b**,**c**) independent experiments.

stabilized Foxo3a-A3 was able to induce secondary recombination in BCR$^+$ cells. SLP-65–deficient BCR$^+$ cells expressing the recombination reporter were transduced with empty vector (IRES-tomato) or a vector encoding Foxo3a-A3 (Foxo3a-A3–IRES–tomato). GFP expression, as a marker for recombination, and cell size in tomato$^-$ and tomato$^+$ populations were analyzed by flow cytometry after 48 h. As in pre-BCR$^+$ cells, expression of the stabilized mutant of Foxo3a in BCR$^+$ cells induced a reduction in cell size, indicating a halt in proliferation (**Fig. 8c**). This cell cycle arrest was accompanied by the recombination of the artificial substrate, resulting in a strong increase in the proportion of GFP$^+$ cells compared to the control vector–transduced population (**Fig. 8c**). Like cells treated with Ly294002, GFP$^+$ cells transduced with the Foxo3a-A3 vector contained populations expressing reduced and increased κ light chain surface expression (**Fig. 8c**). This finding suggests that PKB inhibits secondary light chain recombination by repressing Foxo3a activity and that Foxo3a, and most likely Foxo1, are key regulators of secondary light chain recombination and receptor editing.

On the basis of these data, we propose a model for the regulation of primary and secondary light chain recombination in pre-B and immature B cells (**Supplementary Fig. 4** online). In the absence of SLP-65 activity, autonomous pre-BCR signaling activates Syk, which triggers the PI(3)K-PKB pathway. Once activated, PKB phosphorylates Foxo proteins, promoting their nuclear export and degradation. As a consequence of the inactivation of Foxo proteins, light chain recombination and cellular differentiation are suppressed. In the presence of SLP-65, however, activity of PKB is reduced, leading to nuclear localization and stabilization of Foxo proteins. The latter scenario applies to cells that express a functional pre-BCR, as SLP-65–dependent pre-BCR signals promote primary light chain recombination. Similarly affected are immature B cells that lack BCR expression owing to non-productively rearranged light chain genes; in this case Foxo proteins are not inactivated by PKB and can therefore induce further light chain rearrangements. Similarly, in immature B cells with light chain rearrangements that result in the expression of an autoreactive BCR, strong BCR signals transduced through SLP-65 inhibit PKB activity and activate Foxo proteins and secondary light chain recombination. However, in immature B cells expressing a non-autoreactive BCR, tonic signals result in stable PI(3)K-PKB activation and Foxo protein inactivation.

DISCUSSION

Roles for PI(3)K, PKB and Foxo transcription factors in cellular differentiation have already been proposed, as many leukemic cells exhibit constitutive activation of the PI(3)K-PKB pathway[22,33]. In cells transformed by oncogenic BCR-Abl, signaling through PI(3)K and PKB is crucial for maintenance of the leukemic phenotype and has been shown to result in constitutive phosphorylation and inactivation of Foxo3a[34–36]. Notably, ectopic expression of an active Foxo3a mutant is sufficient to induce differentiation of BCR-Abl transformed cells[27], suggesting that inhibition of Foxo3a transcriptional activity by PI(3)K-PKB is critical for development of leukemia. Further evidence for a function of Foxo transcription factors in regulating proliferation comes from Foxo-deficient mice. Single knockout mice do not show tumor-prone phenotypes, probably because of redundancy among Foxo family members[16,31,37]. Indeed, recent data show that the concerted deletion of Foxo1, Foxo3a and Foxo4 promotes the development of lymphomas and other tumors, demonstrating that Foxo transcription factors function as tumor suppressors[18].

The precise mechanism underlying the effect of Foxo3a on pre-B cell differentiation needs to be clarified, but it is at least partially based on induction of the recombination machinery. RAG-2 normally accumulates during the G1 phase of the cell cycle and is rapidly degraded upon entry into S phase, when Cdk2–cyclin A complexes phosphorylate RAG-2 and thereby mark it for ubiquitination and proteasomal degradation[29,30,38]. We found that Foxo3a-A3 expression counteracted proliferation of pre-B cells and enabled GFP–RAG-2 accumulation. This observation supports previous data showing that Foxo3a regulates the transcription of several cell cycle–related genes[39,40]. However, as p27-induced cell cycle arrest *per se* was not sufficient to induce light chain recombination, it is also possible that Foxo3a promotes light chain rearrangements by transcriptional activation of other genes directly involved in recombination, such as the light chain gene segments themselves. Such targets of Foxo3a have not yet been reported.

Studies with receptor-deficient pre-B cells indicated that PKB, Foxo3a and Foxo1 are regulated by autonomous signaling, as pre-BCR expression alone was sufficient to activate PKB, promote proliferation and counteract light chain recombination. As constitutively active Syk also inhibits differentiation of pre-B cells, Syk may transduce these autonomous pre-BCR signals[22]. A known target of Syk-mediated signaling is CD19, which participates in the binding and activation of PI(3)K and accounts for most PI(3)K activity and PKB phosphorylation induced by BCR ligation[41,42]. Evidence for an involvement of these components in autonomous signaling comes from knockout mouse studies, in which B cell development in mice deficient for Syk, CD19 or PI(3)K subunits was found to be blocked at the pro-B to pre-B cell transition[23,43–45].

The fact that pre-BCR signals suppress light chain recombination in SLP-65–deficient pre-B cells raises the question of how Foxo3a can be released from its PKB-mediated inhibition. Our data show that SLP-65 activity reduces PKB phosphorylation and thereby counteracts the continuous PKB-mediated inactivation of Foxo3a. This finding supports the published role of SLP-65 as a tumor suppressor and explains how SLP-65–independent pre-BCR signals induce proliferation, whereas SLP-65–dependent signals direct cells toward differentiation[5]. Expression of SLP-65 in SLP-65–deficient cells downregulates the pre-BCR, suggesting that the loss of PKB activity is merely the result of reduced receptor expression[5]. It is also possible, however, that SLP-65 reduces PKB phosphorylation by directly interfering with autonomous receptor signaling and PI(3)K activation, probably through Syk and/or CD19. Alternatively, SLP-65 may counteract PKB through the activation of lipid phosphatases such as PTEN and SHIP, which antagonize PI(3)K by dephosphorylation of phosphatidylinositol-3,4,5-trisphosphate to phosphatidylinositol-4,5-bisphosphate.

Like pre-BCR–mediated signaling, BCR-mediated tonic signaling was sufficient to activate the PI(3)K-PKB pathway, suggesting that light chain recombination in pre-B and immature B cells is regulated in a similar manner. Indeed, concerted BCR stimulation and SLP-65 activation strongly reduced PKB and Foxo3a phosphorylation in immature B cells. Notably, our results show that SLP-65 function is essential for Foxo3a activation, as pre-BCR or BCR signaling failed to induce efficient Foxo3a activation in the absence of SLP-65. A role for SLP-65 in regulating secondary light chain recombination is supported by experiments showing impaired receptor editing in SLP-65–deficient cells[11]. Moreover, recent data demonstrate that PLCγ2, which is activated through SLP-65, promotes receptor editing in autoreactive B cells[10].

The importance of PI(3)K signaling for regulating light chain recombination in immature B cells has already been described. Inhibition of PI(3)K in immature B cells results in a 'back-differentiation' to a pro-/pre-B cell–like stage that is accompanied by reexpression

of RAG-2 protein and new light chain rearrangements[8]. Likewise, upregulation of *Rag1* and *Rag2* mRNA upon inhibition of PI(3)K is in agreement with the observation that immature B cells lacking the p85α regulatory subunit of PI(3)K do not suppress RAG expression and that they undergo light chain editing even without BCR stimulation[13]. These findings suggest that basal BCR signals transduced through PI(3)K suppress secondary light chain recombination in non-autoreactive BCR^+ cells. Of note, basal BCR signals in these experiments are transduced through CD19, the main activator of PI(3)K. Data showing that CD19-deficient immature B cells fail to become positively selected and encounter intensive receptor editing further emphasize the role of PI(3)K and basal BCR signaling in suppressing light chain recombination[46,47]. Our results suggest that the main targets of this basal BCR signaling are Foxo proteins, as, like PI(3)K inhibition, expression of a stable mutant of Foxo3a in BCR^+ cells resulted in a reduction in cell size and activated recombination machinery.

Together, our results have identified Foxo3a and Foxo1 as important components regulating differentiation and light chain recombination in B cell precursors. Moreover, the potential role of Foxo3a in apoptosis suggests that Foxo proteins may regulate both of the most important processes of central tolerance, namely receptor editing and clonal deletion.

METHODS

Mice. *Foxo3*$^{f/f}$ mice and *Cd79a-Cre* mice have been described previously[31,32]. Animal experiments were performed in compliance with guidelines of the German law and the Max Planck Institute for Immunobiology.

Cell culture. The SLP-65–deficient pre-B cell line 74, the SLP-65–deficient, immunoglobulin-α–deficient pro/pre-B cell line 3046 and the SLP-65–deficient, RAG-2–deficient, λ5-deficient pre-B cell line (TKO) have been described previously[25,48]. Cell lines were established by culturing bone marrow cells of the respective mice for extended times (>6 months) in IL-7–supplemented medium. BCR^+, SLP-65–deficient cell lines were either obtained from spontaneously differentiated 74 cells in the presence of IL-7 or generated by retroviral transduction of 74 cells with a construct encoding c-myc and subsequent IL-7 withdrawal. Upon IL-7 withdrawal, $c\text{-}myc^+$ cells survive as BCR^+ cells, grow indefinitely without IL-7 and can serve as a model system for immature BCR^+ cells.

Cells were cultured in Iscove's medium (Biochrom) containing 10% heat-inactivated FCS (Vitromex), 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin (Invitrogen) and 5×10^{-5} M 2-mercaptoethanol. For IL-7–dependent growth, the supernatant of J558L cells stably transfected with a vector encoding mouse IL-7 was supplemented in excess. For induction of ERT2-fusion proteins, the respective cells were incubated and stimulated with 1 μM 4-OHT (Merck Biosciences). As a negative control, cells were treated with the same volume of ethanol, the solvent of 4-OHT. For inhibition of PI(3)K, cells were treated with 10 μM Ly294002 (Merck Biosciences) for the indicated times.

Plasmids and retroviral transduction. The recombination reporter plasmid and plasmids for expression of μ heavy chain, λ light chain and λ5 have already been described[22,25]. The constitutively active mutant of PKB was generated by cloning a cDNA encoding a myristoylated PKB[24] (provided by B.A. Hemmings; see Acknowledgments) into the bicistronic retroviral vector pMIG (provided by W.S. Pear). For the inducible PKB (pMIG-ERT2-PKB), a cDNA encoding an ERT2 domain followed by a cDNA encoding the N terminus of SLP-65 and full-length PKB was ligated into the retroviral vector backbone. Previous data show that the N terminus targets SLP-65 to the membrane[49] and presumably functions as a motif for membrane recruitment. pMIG-Foxo3a-A3 was generated by PCR amplification of the cDNA encoding the Foxo3a-A3 mutant from pcDNA3-Foxo3a-A3-ER (provided by B.M.T. Burgering) and ligation of the fragment into pMIG. For pMOWS-GFP-RAG-2, RAG-2 cDNA was amplified from a bone marrow cDNA library and cloned in-frame with a cDNA encoding GFP into the retroviral vector pMOWS. The pMIG-based vector pMItom was generated by amplification of the region encoding the red fluorescent protein tdTomato[50] (provided by R.Y. Tsien) by PCR. The fragment was cloned into pMIG to replace the GFP-coding region. pMItom-Foxo3a-A3 and pMItom-ERT2-SLP-65 were generated by cloning fragments encoding full-length Foxo3a-A3 and ERT2-SLP-65 into the multiple cloning site of pMItom. The vector pMImyrG, used for cell cycle analysis, was generated by inserting a myristoylation signal 5′ of the GFP cDNA in pMIG. Pre-B cells were retrovirally transduced as described previously[48]. In short, the Phoenix retroviral producer cell line was transfected according to the manufacturer's instructions using GeneJuice (Novagen). Retroviral supernatants were harvested after 36 and 60 h. For the transduction, pre-B cells were mixed with supernatants and centrifuged at 300*g* at 37 °C for 3 h. Transduced cells were cultured either with or without IL-7 and analyzed as indicated for the respective experiment.

Flow cytometry. Aliquots of cells were stained for flow cytometry on FACSCalibur and LSRII flow cytometers (Becton Dickinson) using Cy5–anti-IgM (μ chain-specific, Southern Biotechnology), biotin–anti-κ (Southern Biotechnology), phycoerythrin–anti-B220 (Southern Biotechnology), phycoerythrin–anti-κ (BD Biosciences) and Cy5-streptavidin (Dianova).

RT-PCR. Total RNA was isolated from pre-B cell lines using Trizol reagent (Invitrogen). The synthesis of cDNA was performed as previously described[25]. For the amplification of *Hprt1* cDNA, 5′-GCTGGTGAAAAGGACCTCT-3′ and 5′-CACAGGACTAGAACACCTGC-3′ were used as primers. The cDNA for *Rag1* was amplified with 5′-TGCAGACATTCTAGCACTCTGG-3′ and 5′-ACATCTGCCTTCACGTCGAT-3′ primers and the cDNA for *Rag2* with 5′-CACATCCACAAGCAGGAAGTACAC-3′ and 5′-TCCCTCGACTATACACC ACGTCAA-3′ primers.

Cell cycle analysis. Transduced cells were resuspended in 200 μl cold PBS and added slowly with vortexing to a flow cytometry tube containing 4 ml ice-cold 70% ethanol. After incubation on ice for 12 h, the fixed cells were centrifuged (460*g*, 10 min), resuspended in 1 ml propidium iodide staining solution (10 μg/ml RNaseA, 40 μg/ml propidium iodide in PBS) per sample and incubated at 37 °C for 30 min. Stained cells were washed once with PBS and analyzed by flow cytometry. Cell cycle kinetics were calculated using FlowJo software (Tree Star).

Cell stimulation and immunoblotting. NIP-specific BCR^+ cells (1.5×10^6 cells/ml) were stimulated with 2 μg/ml NIP-conjugated BSA (15 haptens per BSA molecule; Biosearch Technologies) at 37 °C for the lengths of time indicated for the individual experiments. For immunoblotting, 1.5×10^6 cells per sample were harvested and lysed in 50 μl modified RIPA buffer (50 mM Tris HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, pH 8, 1 mM sodium orthovanadate, 1 mM NaF and protease inhibitor cocktail (Sigma-Aldrich)). Lysates were separated on 10% SDS-polyacrylamide gels and transferred to PVDF membranes (Millipore). Membranes were blocked with 5% dry milk in PBT (PBS, 0.1% Tween-20) for 2 h at room temperature with constant agitation. Primary antibodies were diluted in PBT supplemented with 4% BSA fraction V (BIOMOL Research Laboratories); secondary antibodies were diluted in blocking solution. Immunoreactive proteins were detected using a chemoluminescence detection system (ECL; GE Healthcare). Antibodies used were anti-phospho-Foxo3a Thr32 (Upstate Biotechnology), anti-phospho-Foxo1 Ser256 (Cell Signaling Technologies), anti-phospho-AKT Ser473 (193H12, Cell Signaling Technologies), anti-AKT (Cell Signaling Technologies) and anti-actin (I-19, Santa Cruz Biotechnology).

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

S.H. designed experiments, did all experimental studies unless otherwise indicated and wrote the manuscript. E.H. established the correlation between receptor expression and PKB regulation. S.M. established the inducible PKB (ERT2-PKB) system and performed the respective experiment. J.-H.P. and R.A.D. provided suggestions and mice deficient for Foxo3a. M.R. provided suggestions for experimental design. H.J. designed experiments, supervised the study, developed the concept and wrote the manuscript together with S.H.

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