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Sin1-mTORC2 Suppresses *rag* and *il7r* Gene Expression through Akt2 in B Cells

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SUMMARY

Mammalian target of rapamycin (mTOR) is an important mediator of phosphoinositol-3-kinase (PI3K) signaling. PI3K signaling regulates B cell development, homeostasis, and immune responses. However, the function and molecular mechanism of mTOR-mediated PI3K signaling in B cells has not been fully elucidated. Here we show that Sin1, an essential component of mTOR complex 2 (mTORC2), regulates B cell development. Sin1 deficiency results in increased IL-7 receptor (*il7r*) and RAG recombinase (*rag1* and *rag2*) gene expression, leading to enhanced pro-B cell survival and augmented V(D)J recombinase activity. We further show that Akt2 specifically mediates the Sin1-mTORC2 dependent suppression of *il7r* and *rag* gene expression in B cells by regulating FoxO1 phosphorylation. Finally, we demonstrate that the mTOR inhibitor rapamycin induces *rag* expression and promotes V(D)J recombination in B cells. Our study reveals that the Sin1/mTORC2-Akt2 signaling axis is a key regulator of FoxO1 transcriptional activity in B cells.

INTRODUCTION

Mammalian TOR is a constitutively expressed, evolutionarily conserved protein kinase that plays a central role in the regulation of cell growth, proliferation, apoptosis, and metabolism (Wullschleger et al., 2006). Mammalian TOR resides in two distinct protein complexes termed mammalian TOR complex 1 (mTORC1) and mammalian TORC2 (mTORC2) (Guertin and Sabatini, 2009; Wullschleger et al., 2006). mTORC1 contains mTOR, raptor, mLST8 (GβL), and PRAS40, and its function is acutely inhibited by rapamycin, a potent immunosuppressant with antitumor effect (Guertin and Sabatini, 2009; Wullschleger et al., 2006). Upon stimulation by nutrients, growth factors, hormones, and energy signals, mTORC1 phosphorylates the translational regulators S6K and 4EBP1, which leads to increased cellular protein synthesis and ribosome biogenesis (Gingras et al., 2004; Harris and Lawrence, 2003; Wullschleger et al., 2006). mTORC2 contains Rictor, Sin1, and mLST8 in addition to mTOR, and regulates actin polymerization and cytoskeleton function (Guertin and Sabatini, 2009; Wullschleger et al., 2006). Mammalian TORC2 is resistant to acute rapamycin inhibition. However, chronic rapamycin exposure also inhibits mTORC2 in vitro and in vivo (Facchinetti et al., 2008; Sarbassov et al., 2006; Zeng et al., 2007). Recent studies show that mTORC2 regulates Akt/PKB in both a PI3K-dependent and PI3K-independent manner (Facchinetti et al., 2008; Jacinto et al., 2006; Sarbassov et al., 2005).

Akt/PKB is one of the most-studied members of the AGC kinase family, which also includes S6K, RSK, SGK, and PKC (Peterson and Schreiber, 1999; Woodgett, 2005). Like most members in this family, Akt is phosphorylated at two key residues that are located in the catalytic center (activation loop or T loop) and the C-terminal hydrophobic motif (HM). Phosphorylation of Akt/PKB at the T loop site (Thr308) is mediated by PDK1 and is essential for Akt catalytic activity (Alessi et al., 1997; Stephens et al., 1998). Phosphorylation of Akt at the HM site (Ser473) is independently mediated by mTORC2 (Jacinto et al., 2006; Sarbassov et al., 2005). Although Akt Ser473 phosphorylation is widely used as an indicator of Akt activation, the precise physiological function of this phosphorylation is still not fully understood. Phosphorylation at the Akt HM site may facilitate the PDK1-mediated phosphorylation of the T loop site, thereby enhancing Akt activity upon growth factor stimulation and PI3K activation (Alessi et al., 1996; Biondi, 2004; Scheid et al., 2002). Surprisingly however, genetic studies reveled that mTORC2 disruption, which abolishes Akt HM site phosphorylation, does not inhibit T loop phosphorylation (Jacinto et al., 2006). Rather, Akt HM site phosphorylation regulates the substrate specificity of Akt (Jacinto et al., 2006). More recently, mTORC2 was shown to phosphorylate Akt at the turn motif (TM) residue Thr450, which controls Akt protein stability (Facchinetti et al., 2008).

B lymphocyte development is divided into distinct stages where immunoglobulin (Ig) variable (V), diversity (D), and joining (J) genes of the Ig heavy (IgH) chain and V and J genes of the Ig

light (IgL) chain undergo somatic recombination, generally referred to as V(D)J recombination, to generate the B cell antigen receptor (BCR) (Schatz et al., 1989; Schlissel, 2003; Spicuglia et al., 2006). V(D)J recombination is mediated by the recombination activation genes *rag1* and *rag2*, which associate and form the V(D)J recombinase (Leu and Schatz, 1995; Schatz et al., 1989). IgH gene recombination occurs first in progenitor B (pro-B) cells and, if successful, leads to the expression of the pre-BCR. Pre-BCR signals provide critical feedback about the functionality of the IgH chain, allowing only those developing B cells with a functional pre-BCR to further differentiate into precursor B (pre-B) cells and begin IgL gene rearrangement (Herzog et al., 2009; Martensson et al., 2007). Pre-BCR signals promote cell survival and proliferation and suppress *rag* expression to prevent further IgH recombination (Geier and Schlissel, 2006; Schlissel, 2003). The pre-BCR-dependent suppression of RAG expression contributes to allelic exclusion of IgH genes, terminates additional V(D)J recombination that could disrupt a productively rearranged IgH gene, and prevents aberrant V(D)J recombination that may result in genomic instability in proliferating pre-B cells. Subsequent IgL recombination leads to the expression of the BCR on immature B cells.

PI3K and Akt negatively regulate RAG expression and V(D)J recombination (Amin and Schlissel, 2008; Llorian et al., 2007; Verkoczy et al., 2007). The Forkhead family transcription factor FoxO1 is a direct regulator of *rag* genes downstream of PI3K and Akt (Amin and Schlissel, 2008; Dengler et al., 2008; Herzog et al., 2008, 2009). Genetic or pharmacological inhibition of the PI3K pathway in B cells increases the expression of FoxO1 target genes and results in abnormal B cell function (Donahue and Fruman, 2004; Llorian et al., 2007; Suzuki et al., 1999; Verkoczy et al., 2007). These studies suggest that Akt may mediate PI3K signaling to control FoxO1 activity in B cells; however, it is unclear how PI3K signals are integrated through Akt to regulate FoxO1 and it is not known if Akt is the sole mediator of PI3K-dependent signals that regulate FoxO1. Additionally, the Akt isoform that regulates FoxO1 phosphorylation and function in B cells has not been identified and the molecular mechanism through which pre-BCR/BCR signals activate Akt and suppress FoxO1 activity is unknown.

In this study, we reveal the function of mTORC2 in B cells and elucidate mechanisms of mTORC2 regulation of B cell development. We show that genetic ablation of Sin1 in mice disrupts mTORC2 and abolishes Akt phosphorylation at Ser473 and Thr450 but not at Thr308 in developing B cells. Developing *Sin1*$^{-/-}$ B cells show increased IL-7 receptor expression, enhanced response to IL-7, augmented RAG expression, and elevated V(D)J recombinase activity. We demonstrate that the mTORC2-dependent Akt HM phosphorylation is specifically required for the suppression of *rag* gene expression and FoxO1 phosphorylation is dependent on both Sin1 and Akt2 in B cells. Finally, we show that the mTOR inhibitor rapamycin increases *rag1* expression and promotes V(D)J recombination in B cells. These data reveal that the Sin1/mTORC2-Akt2 signaling axis regulates IL-7 responsiveness, RAG expression, and V(D)J recombination in developing B cells.

RESULTS

Sin1 Regulates B Cell Development

To investigate the function of Sin1 in vivo, we generated Sin1 KO mice (Jacinto et al., 2006). The *Sin1*$^{-/-}$ embryos die during gestation between embryonic day (E) 10.5 and 15.5 due to severe defects in cardiovascular development (this will be described in another study). We successfully reconstituted lethally irradiated CD45.1 congenic mice with wild-type and *Sin1*$^{-/-}$ fetal liver hematopoietic cells from E11.5–E12.5 embryos, demonstrating that *Sin1*$^{-/-}$ hematopoietic stem cells are capable of reconstituting the hematopoietic system of adult mice (data not shown).

We examined bone marrow of chimeric mice and found that the proportion of immature IgM$^+$ bone marrow B cells was reduced in *Sin1*$^{-/-}$ chimeric mice when compared to *Sin1*$^{+/+}$ chimeras (Figure 1A). Specifically, in a representative pair of *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ chimeric mice, 70% (12% CD19$^+$IgM$^+$ / 17% CD19$^+$) of the bone marrow B cells were IgM$^+$, while only 43% (3% CD19$^+$IgM$^+$ / 7% CD19$^+$) of *Sin1*$^{-/-}$ bone marrow B cells were IgM$^+$. We also analyzed a chimeric mouse that contained a 1:1 ratio of host (wild-type) to *Sin1*$^{-/-}$ donor cells, allowing us to directly compare developing *Sin1*$^{-/-}$ B cells and wild-type B cells within the same animal. The proportion and number of *Sin1*$^{-/-}$ IgM$^+$ B cells was reduced by approximately 3-fold when compared to the wild-type IgM$^+$ B cells (Figures 1B and 1C). We also observed a 4-fold increase in the total number of *Sin1*$^{-/-}$ IgM$^-$ bone marrow B cells when compared to the wild-type IgM$^-$ B cells. The CD19$^+$IgM$^-$ population contains pro-B cells, which are the most immature bone marrow B cell population that also express the pro-B cell surface marker CD45R (B220$^+$) and high levels of CD43 (CD43^{hi}). Analysis of IgM$^-$ bone marrow B cells from the *Sin1*$^{-/-}$ chimeric mice revealed a 2-fold increase in the proportion of *Sin1*$^{-/-}$ B220$^+$CD43^{hi}IgM$^-$ pro-B cells relative to wild-type cells (Figure 1D). Together, these data show that the developmental defect is intrinsic to the *Sin1*$^{-/-}$ B cells and Sin1 deficiency results in expansion of pro-B cells.

Sin1 Deficiency Perturbs B Cell Development In Vitro

We established primary pro-B cell lines from the fetal livers of paired E12.5 *Sin1*$^{-/-}$ and *Sin1*$^{+/+}$ littermate embryos using OP9 stromal cells supplemented with IL-7 (Vieira and Cumano, 2004). We generated four independent pairs of wild-type and *Sin1*$^{-/-}$ pro-B cell lines and observed no defect in the ability in *Sin1*$^{-/-}$ fetal liver hematopoietic cells to give rise to pro-B cells when compared to the wild-type fetal liver hematopoietic cells (Figures S1A–S1C).

To determine the differentiation potential of *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells, we differentiated those cells in vitro on OP9 cells in the absence of IL-7 for 7 days. We found that *Sin1*$^{-/-}$ pro-B cells differentiated in vitro gave rise to a smaller proportion of IgM$^+$ cells than the wild-type cells (Figure 1E). We also observed 3-fold more Igμ^- pro-B cells in the *Sin1*$^{-/-}$ culture than in the *Sin1*$^{+/+}$ B cell culture (Figure 1F). Together, these results show that Sin1 is required for the proper development of IgM$^+$ B cells and suggest that *Sin1* deficiency may enhance the survival and/or proliferation of pro-B cells when IL-7 is limiting.

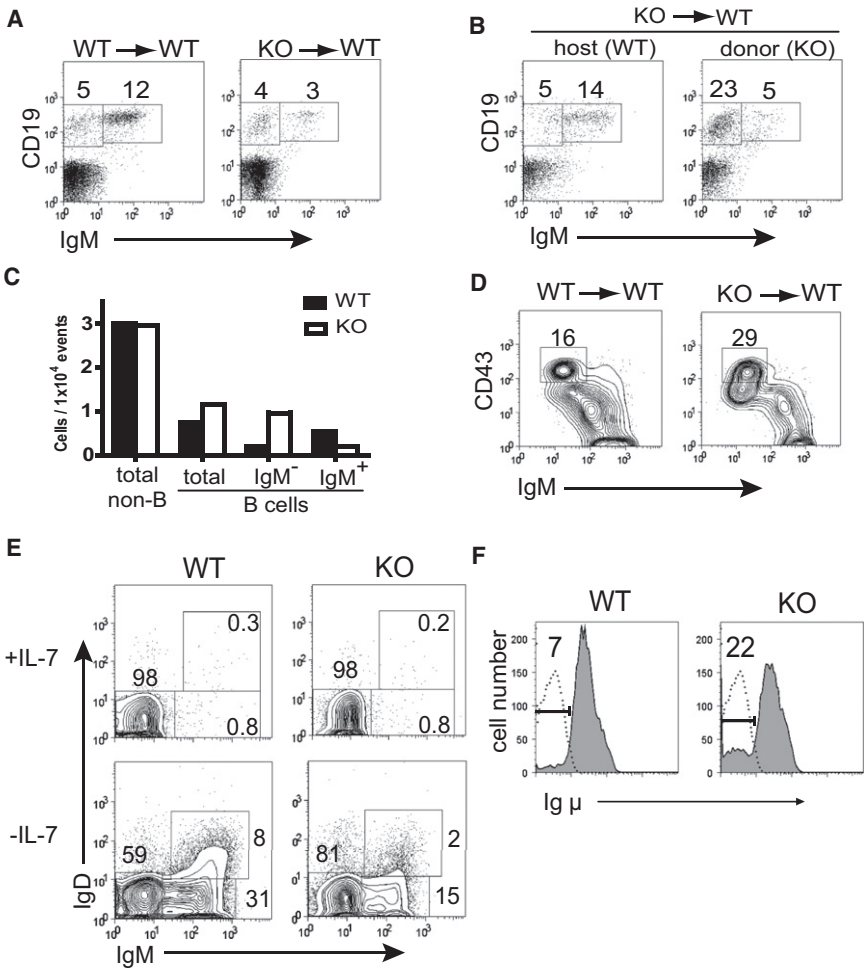

Figure 1. Sin1 Regulates B Cell Development

(A) Bone marrow from *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ chimeric mice was analyzed by flow cytometry and the percentage of CD19^{+} IgM^{-} and CD19^{+} IgM^{+} B cells is indicated. The data are representative of *Sin1*$^{+/+}$ (n = 3) and *Sin1*$^{-/-}$ (n = 4) fetal liver chimeric mice.

(B) Bone marrow from a chimeric mouse containing a 1:1 ratio of wild-type host and *Sin1*$^{-/-}$ donor cells was analyzed by flow cytometry. The plots shown are pregated on CD45.1^{+} host cells or CD45.1^{-} *Sin1*$^{-/-}$ donor cells, and the percentage of CD19^{+}IgM^{-} and CD19^{+}IgM^{+} B cells is indicated.

(C) Bar graph illustrating the total number of *Sin1*$^{+/+}$ host or *Sin1*$^{-/-}$ donor bone marrow cells from the chimeric mouse shown in (B).

(D) The proportion of B220^{+}CD43^{hi}IgM^{-} bone marrow pro-B cells from wild-type or *Sin1*$^{-/-}$ fetal liver chimeric mice. The plots shown are pregated on CD45.2^{+}B220^{+} donor cells and are representative of *Sin1*$^{+/+}$ (n = 3) and *Sin1*$^{-/-}$ (n = 4) fetal liver chimeric mice.

(E) *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells were cultured in vitro on OP9 cells with or without IL-7 for 7 days and surface IgM and IgD expression was analyzed. The plots are representative of four independent experiments.

(F) *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells were cultured on OP9 cells without IL-7 for 7 days, fixed, permeablized, and stained for Igμ chain expression (shaded area). The proportion of Igμ^{-} pro-B cells is indicated. *Rag1*$^{-/-}$ pro-B cells are used as a negative control for Igμ staining (dotted line). Representative plots are shown from three independent experiments.

Sin1$^{-/-}$ Pro-B Cells Exhibit Increased IL-7Rα Expression and Enhanced IL-7-Dependent Survival

IL-7 provides the primary pro-B cell survival signal. Since we observed an increased proportion of *Sin1*$^{-/-}$ pro-B cells in the bone marrow and in the OP9 coculture differentiation assay, we speculated that Sin1 deficiency might enhance pro-B cell responsiveness to IL-7. We examined IL-7 receptor (IL7R) expression in *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells and found that *il7r* mRNA levels were increased approximately 2-fold in *Sin1*$^{-/-}$ pro-B cells relative to *Sin1*$^{+/+}$ pro-B cells (Figure 2A). The expression of membrane bound IL-7R was also increased on *Sin1*$^{-/-}$ pro-B cells when compared to the *Sin1*$^{+/+}$ pro-B cells (Figure 2B). IL-7R was not expressed on *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ IgM^{+} immature B cells indicating that Sin1 only regulates *il7r* expression in pro-B cells (Figure S2).

These data suggested that the increased IL-7R expression on *Sin1*$^{-/-}$pro-B cells may render these cells more sensitive to IL-7 than *Sin1*$^{+/+}$ pro-B cells. Indeed, we found that the number of *Sin1*$^{-/-}$B cells recovered at each time point after IL-7 withdraw was substantially greater than that of *Sin1*$^{+/+}$ cells (Figure 2C). Most notably, the number of viable *Sin1*$^{+/+}$ B cells decreased by approximately 90% 72 hr after IL-7 withdraw while the number of viable *Sin1*$^{-/-}$ B cells at 72 hr was similar to the number of cells initially plated at time 0. These data suggest that *Sin1*$^{-/-}$ pro-B cells exhibit enhanced survival to the IL-7 produced by the OP9 cells.

To further confirm that *Sin1*$^{-/-}$ pro-B cells exhibit enhanced IL-7 responsiveness, we measured IL-7-dependent survival of *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ pro-B cells in the absence of OP9 cells. *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells were washed and cultured in medium containing a low concentration of IL-7 (0.1 ng/ml). The number of live cells was measured at 10 hr or 24 hr after plating. We observed significantly more live *Sin1*$^{-/-}$ pro-B cells than wild-type pro-B cells after 10 hr in culture (Figure 2D). There was little difference between *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ cell viability by 24 hr, indicating that IL-7 is absolutely required for the survival of both wild-type and *Sin1*$^{-/-}$ pro-B cells. We also cultured pro-B cells in medium supplemented with a high concentration of IL-7 (5 ng/ml) and found that *Sin1*$^{-/-}$ pro-B cells showed enhanced survival over *Sin1*$^{+/+}$ pro-B cells under these conditions (Figure 2E). Together, these data show that *Sin1* deficient pro-B cells have increased IL-7 receptor expression and exhibit enhanced IL-7-dependent survival.

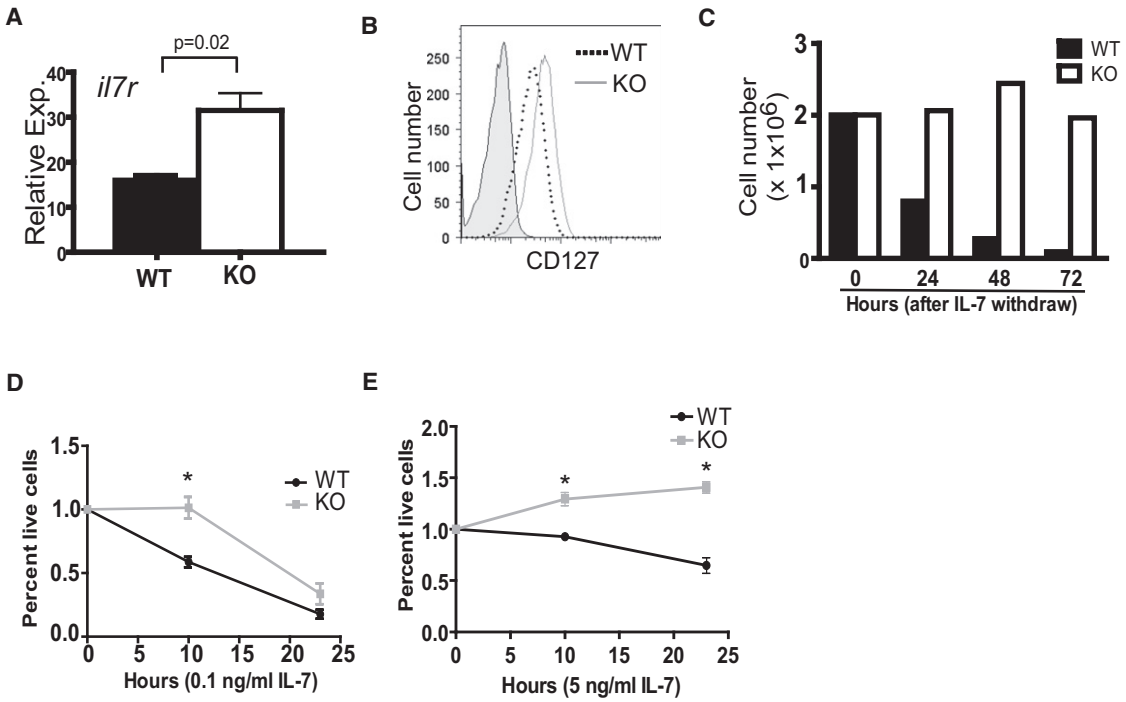

Figure 2. *Sin1*$^{-/-}$ Pro-B Cells Exhibit Increased *il7r* Expression and Enhanced IL-7-Dependent Survival

(A) IL-7 receptor (*il7r*) mRNA levels in *Sin1*$^{+/+}$ (WT) or *Sin1*$^{-/-}$ (KO) pro-B cells were measured by qPCR and normalized to *GAPDH* expression. Samples were run in triplicate and data are representative of two independent experiments.

(B) Expression of IL-7R (CD127) on *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells was measured by flow cytometry. Shaded area is the isotype control staining. The plots are representative of three independent experiments.

(C) *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells were cultured on OP9 cells without exogenous IL-7. The total number of live cells recovered at each time point was measured. Dead cells were excluded from the analysis by trypan blue staining. Data are representative of two independent experiments.

(D) *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells were cultured without OP9 cells in medium supplemented with 0.1 ng/ml IL-7. The number of live cells at 10 and 24 hr after plating was determined by trypan blue dye exclusion. Each data point represents triplicate wells from one of two independent experiments (* = $p < 0.01$).

(E) *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells were cultured without OP9 cells in medium supplemented with 5 ng/ml IL-7. The number of live cells at various time points after plating was determined by trypan blue dye exclusion. Each data point represents triplicate wells from one of two independent experiments (* = $p < 0.01$). Error bars indicate standard deviation.

Sin1$^{-/-}$ B Cells Lack Functional mTORC2 and Exhibit Defective Akt Phosphorylation

To investigate the mechanism of Sin1 function in B cells we established Abelson murine leukemia virus (Ab-MuLV) transformed pre-B cells from *Sin1*$^{+/+}$, *Sin1*$^{+/-}$, and *Sin1*$^{-/-}$ pro-B cells. *Sin1*$^{-/-}$Ab-MuLV pre-B cells expressed B lineage surface markers and showed no defect in growth, proliferation, or survival when compared to *Sin1*$^{+/+}$ or *Sin1*$^{+/-}$ pre-B cells (data not shown). To determine if Sin1 is essential for mTORC2 integrity in B cells, the endogenous mTORC2 was immunoprecipitated from *Sin1*$^{+/-}$ or *Sin1*$^{-/-}$ Ab-MuLV pre-B cells using an anti-Rictor antibody. As expected, mTOR and Sin1 coimmunoprecipitated with Rictor in the *Sin1*$^{+/-}$ but not *Sin1*$^{-/-}$ pre-B cells (Figure 3A). In addition, Rictor and mTOR co-immunoprecipitated with Sin1 in the *Sin1*$^{+/-}$ cells (data not shown). These data show that Sin1 is required for the mTORC2 integrity in B cells. Consistent with these results, phosphorylation of Akt at the mTORC2 target sites Ser473 and Thr450 was abolished in the *Sin1*$^{-/-}$ pro-B cells and Ab-MuLV pre-B cells but not in the Sin1-sufficient B cells (Figure 3B). The Akt T loop (Thr308) phosphorylation was approximately 1.5 fold more in *Sin1*$^{-/-}$ pro-B cells and about 10-fold more in *Sin1*$^{-/-}$ Ab-MuLV pre-B cells than that in *Sin1*$^{+/+}$ pro-B and pre-B cells, respectively (Figure 3B). Furthermore, Akt expression in both primary and transformed *Sin1*$^{-/-}$ B cells was decreased, as well, consistent with our previous studies showing that the TM phosphorylation regulates Akt protein stability (Facchinetti et al., 2008).

Sin1 Is Required for FoxO1 Phosphorylation in B Cells

The FoxO transcription factors are evolutionarily conserved targets of Akt and disruption of mTORC2 results in the selective impairment of FoxO1/3a phosphorylation in embryonic fibroblasts (Jacinto et al., 2006). Therefore, we examined FoxO1 phosphorylation in control and *Sin1*$^{-/-}$ Ab-MuLV pre-B cells and found that FoxO1 phosphorylation at Thr24 was impaired (Figure 3C). The Akt-dependent phosphorylation of FoxO proteins is known to promote the ubiquitination and subsequent proteasome-dependent degradation of FoxO proteins (Plas and Thompson, 2003). Consistently, more FoxO1 protein was detected in *Sin1*$^{-/-}$ Ab-MuLV pre-B cells than the control cells (Figure 3C). FoxO1 Thr24 phosphorylation was also impaired and FoxO1 protein levels were increased in *Sin1*$^{-/-}$pro-B cells

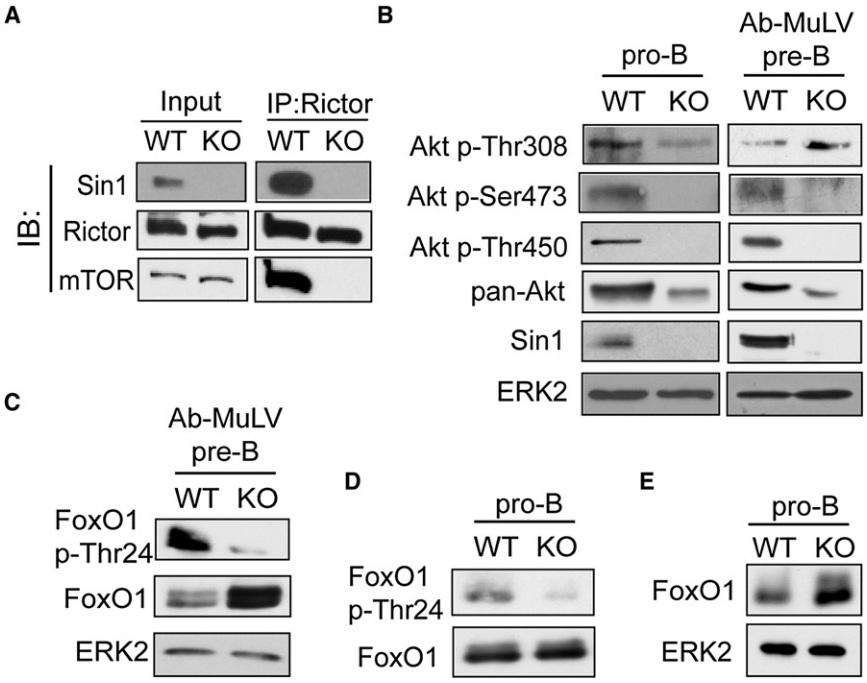

Figure 3. Sin1 Is Required for mTORC2-Dependent Akt Hydrophobic Motif and Akt Turn Motif Phosphorylation in B Cells

(A) The mTORC2 complex integrity in *Sin1*$^{+/-}$ (WT) or *Sin1*$^{-/-}$ (KO) Ab-MuLV pre-B cells was determined by immunoprecipitation (IP) of Rictor followed by immunoblotting (IB) of Sin1, Rictor, and mTOR.

(B) Akt phosphorylation and expression was measured in primary *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells cultured on OP9 cells with IL-7 and Ab-MuLV transformed *Sin1*$^{+/-}$ (WT) or *Sin1*$^{-/-}$ (KO) pre-B cells. Samples were normalized to total cellular protein and blotted as indicated.

(C) FoxO1 phosphorylation and expression was measured in *Sin1*$^{+/-}$ or *Sin1*$^{-/-}$ Ab-MuLV pre-B cells. Samples were normalized to total cellular protein and blotted for FoxO1 or FoxO1 p-Thr24.

(D) FoxO1 phosphorylation was measured in *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells cultured with serum and IL-7. The samples were normalized to total FoxO1 protein then blotted for FoxO1 p-Thr24.

(E) FoxO1 expression was measured in *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells. The samples were normalized to total cellular protein.

when compared to *Sin1*$^{+/+}$ pro-B cells (Figures 3D and 3E). Together, these data show that Sin1 deletion impairs FoxO1 phosphorylation in B developing cells.

Sin1 Suppresses RAG Expression in Developing B Cells

Our observation that FoxO1 phosphorylation is impaired and FoxO1 expression is increased in *Sin1*$^{-/-}$ B cells provides a possible mechanism to explain the elevated expression of *il7r,* which was recently identified as a FoxO1 target gene (Kerdiles et al., 2009). The genes *rag1* and *rag2* are also regulated by FoxO1 in B cells (Amin and Schlissel, 2008; Herzog et al., 2008; Llorian et al., 2007; Verkoczy et al., 2007). We measured *rag1* transcript levels in *Sin1*$^{+/-}$ and *Sin1*$^{-/-}$ Ab-MuLV pre-B cells and found that *rag1* expression was significantly elevated in *Sin1*$^{-/-}$ pre-B cells compared to the *Sin1*$^{+/-}$cells (Figure 4A). *Rag* expression is suppressed by the v-Abl kinase and can be rapidly induced by inhibiting v-Abl activity with Imatinib (Muljo and Schlissel, 2003). We found that Imatinib treatment substantially increased *rag1* expression in both control and *Sin1*$^{-/-}$ pre-B cells. However, *rag1* expression was increased an additional 3-fold in Imatinib treated *Sin1*$^{-/-}$ pre-B cells when compared to the *Sin1*$^{+/-}$ pre-B cells (Figure 4A). In addition, RAG1 protein was readily detectable in *Sin1*$^{-/-}$ but not *Sin1*$^{+/-}$ Ab-MuLV pre-B cells cultured in the absence of Imatinib (Figure 4B).

Next, we examined *rag1* expression in *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells cultured on OP9 cells with IL-7 and found that *rag1* expression was approximately 2-fold more in *Sin1*$^{-/-}$ pro-B cells than in *Sin1*$^{+/+}$ pro-B cells (Figure 4C, at the 0 hr time point). IL-7 attenuates *rag1* expression in pro-B cells (Melamed et al., 1997). Therefore we measured *rag1* expression in *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells 20 hr after IL-7 withdrawal and found that *rag1* expression increased 2-fold in *Sin1*$^{+/+}$ pro-B cells while *rag1* expression showed little increase in *Sin1*$^{-/-}$ pro-B cells (Figure 4C). At 48 hr after IL-7 withdrawal, *Sin1*$^{+/+}$ pro-B cells showed a 5-fold increase in *rag1* expression while *Sin1*$^{-/-}$ pro-B cells showed a 3-fold increase in *rag1* expression relative to the 0 hr time point. These data suggest that IL-7 signals suppress *rag* expression through a mechanism that is not dependent on *Sin1*.

We also examined *rag1* expression in *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pre-B cells and immature B cells differentiated in vitro on OP9 cells. We found that *rag1* expression was 3-fold higher in *Sin1*$^{-/-}$ pre-B cells than *Sin1*$^{+/+}$ pre-B cells and 2-fold higher in *Sin1*$^{-/-}$ immature B cells than *Sin1*$^{+/+}$ immature B cells (Figure 4D). Expression of *rag2* was also higher in both *Sin1*$^{-/-}$ pre-B and immature B cells than that in *Sin1*$^{+/+}$ B cells (Figure 4D). To determine if Sin1 deficiency influences IgL recombination we examined the Igκ and Igλ chain expression on immature IgM^{+} B cells. Analysis of IgL chain expression on immature IgM^{+} *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ B cells revealed a 2-fold increase in the proportion of Igλ light chain expressing *Sin1*$^{-/-}$ B cells when compared to *Sin1*$^{+/+}$ B cells (Figure S3). Together, these results show that Sin1 regulates *rag* expression in B cells and suggest that Sin1 influences IgL recombination in developing B cells.

Finally, we reconstituted the *Sin1*$^{-/-}$ Ab-MuLV pre-B cells with human *Sin1* cDNA and examined RAG1 protein levels in these cells. Restoration of Sin1 expression in *Sin1*$^{-/-}$ Ab-MuLV pre-B cells decreased RAG1 protein expression while a control virus lacking human Sin1 expression did not decrease RAG1 expression (Figure 4E). These data confirm that Sin1 is a negative regulator of *rag* expression in developing B cells.

Sin1 Suppresses V(D)J Recombinase Activity in Developing B Cells

To determine if the elevated *rag* gene expression and RAG1 protein observed in the *Sin1*$^{-/-}$ pre-B cells correlates with

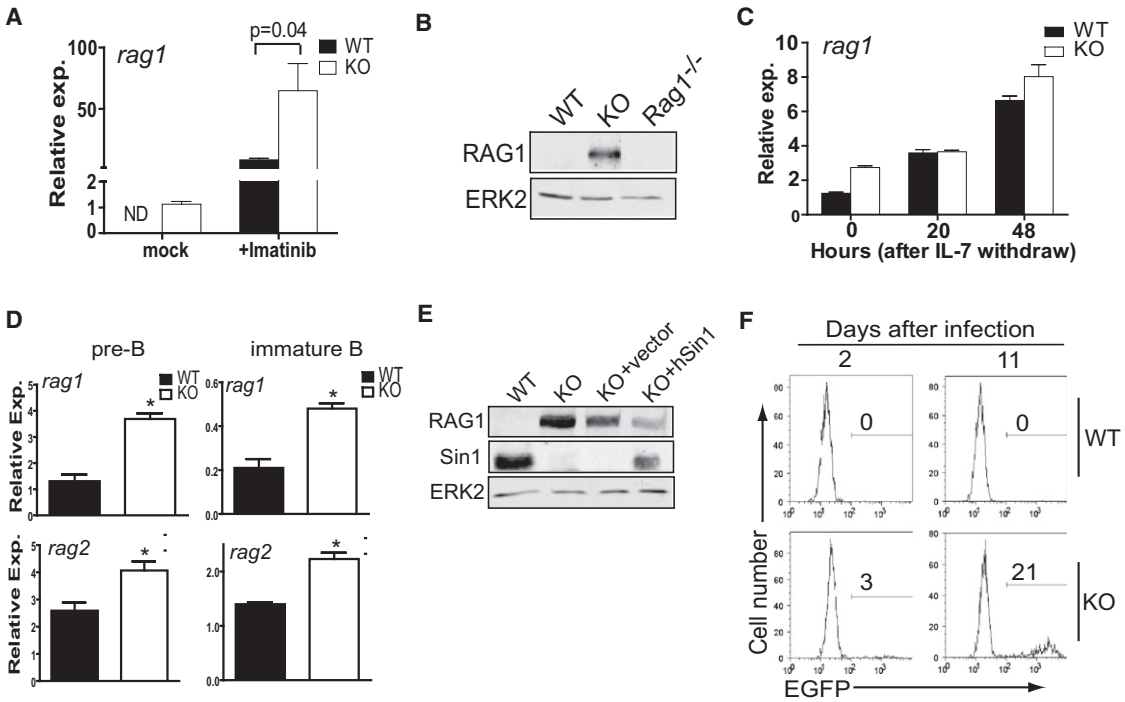

Figure 4. Increased RAG Expression and V(D)J Recombinase Activity in Developing *Sin1*$^{-/-}$ B Cells

(A) *Rag1* mRNA expression was measured by qPCR in *Sin1*$^{+/-}$ (WT) or *Sin1*$^{-/-}$ (KO) Ab-MuLV pre-B cells cultured for 5 hr in the presence or absence of Imatinib. *Rag1* mRNA was normalized to *GAPDH* mRNA. ND: not detected.

(B) RAG1 protein in *Sin1*$^{+/-}$ (WT), *Sin1*$^{-/-}$ (KO) or *rag1*$^{-/-}$ Ab-MuLV pre-B cells was measured by immunoblotting. The ERK2 protein is used as a loading control.

(C) *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells were cultured on OP9 cells without exogenous IL-7 for 0, 20, or 48 hr. *Rag1* expression was measured by qPCR. *Rag1* expression was normalized to *GAPDH* mRNA. Samples were run in triplicate and are representative of two independent experiments.

(D) *Sin1*$^{+/+}$ (KO) or *Sin1*$^{-/-}$ (WT) pro-B cells were differentiated in vitro on OP9 cells for 7 days, and *rag1* or *rag2* expression in pre-B and immature B cells obtained from these cultures was measured by qPCR as described in (A). Samples were run in triplicate and are representative of two independent experiments (*$p < 0.01$).

(E) *Sin1*$^{-/-}$ Ab-MuLV pre-B cells were infected with a retrovirus expressing human Sin1 or a control empty retrovirus, and the RAG1 expression was measured by immunoblotting. Data are representative of two independent experiments.

(F) *Sin1*$^{+/-}$ or *Sin1*$^{-/-}$ Ab-MuLV pre-B cells were infected with a retrovirus containing an EGFP RAG recombinase reporter. The infected Ab-MuLV pre-B cells were then grown under normal culture conditions, and the EGFP^{+} cells were determined by flow cytometry 2 days and 11 days later. The plots shown are gated on infected cells (hCD4^{+} cells). The data are representative of three independent experiments. Error bars indicate standard deviation.

V(D)J recombinase activity, we infected wild-type or *Sin1*$^{-/-}$ Ab-MuLV pre-B cells with a retrovirus containing an EGFP-based V(D)J recombinase reporter. The reporter contains an anti-sense orientated EGFP cDNA flanked by 12- and 23-recombination signal sequences. RAG-mediated recombination flips the EGFP cDNA sequence to the sense orientation permanently marking the cell with EGFP expression. *Sin1*$^{+/-}$ or *Sin1*$^{-/-}$ pre-B cells infected with the reporter virus were assayed 2 days and 11 days postinfection by flow cytometry. The infected cells were first identified by human CD4 expression from an IRES-hCD4 cassette in the retroviral vector and then analyzed for EGFP expression. At day 2, no *Sin1*$^{+/-}$ pre-B cells expressed EGFP while 3% of the *Sin1*$^{-/-}$ hCD4^{+} pre-B cells expressed EGFP. Eleven days after infection, 21% of the hCD4^{+} *Sin1*$^{-/-}$ pre-B cells expressed EGFP while none of the *Sin1*$^{+/-}$ hCD4^{+} pre-B cells expressed EGFP (Figure 4F). These data show that Sin1 deficiency results in increased V(D)J recombinase activity in developing B cells.

Akt2 Regulates FoxO1 Phosphorylation and Suppresses *il7r* and *rag* Expression in B Cells

Our data show that Sin1/mTORC2 regulates the expression of the FoxO1 target genes *il7r*, *rag1*, and *rag2*. Studies from our group and other laboratories suggest that Sin1/mTORC2 mediates PI3K signals to activate Akt, which in turn suppresses FoxO1 activity. However, genetic evidence supporting this model is currently lacking. If this model is correct, we predict that the Sin1 and Akt-deficient B cells will share a common phenotype showing defective FoxO1 regulation and augmented FoxO1 target gene expression. To test this model, we established primary pro-B cell lines from *Akt1*$^{-/-}$, *Akt2*$^{-/-}$, and *Akt1*$^{-/-}$/*Akt2*$^{-/-}$ mice. We first examined *rag* expression in these cells by quantitative RT-PCR, and found that Akt1 deficiency had no effect on *rag1* expression in pro-B cells (Figure 5A). In contrast, the *rag1* expression was elevated approximately 3-fold in *Akt2*$^{-/-}$ pro-B cells relative to the wild-type cells (Figure 5A). Consistent with the increased *rag1* mRNA expression, RAG1 protein levels were also significantly increased in

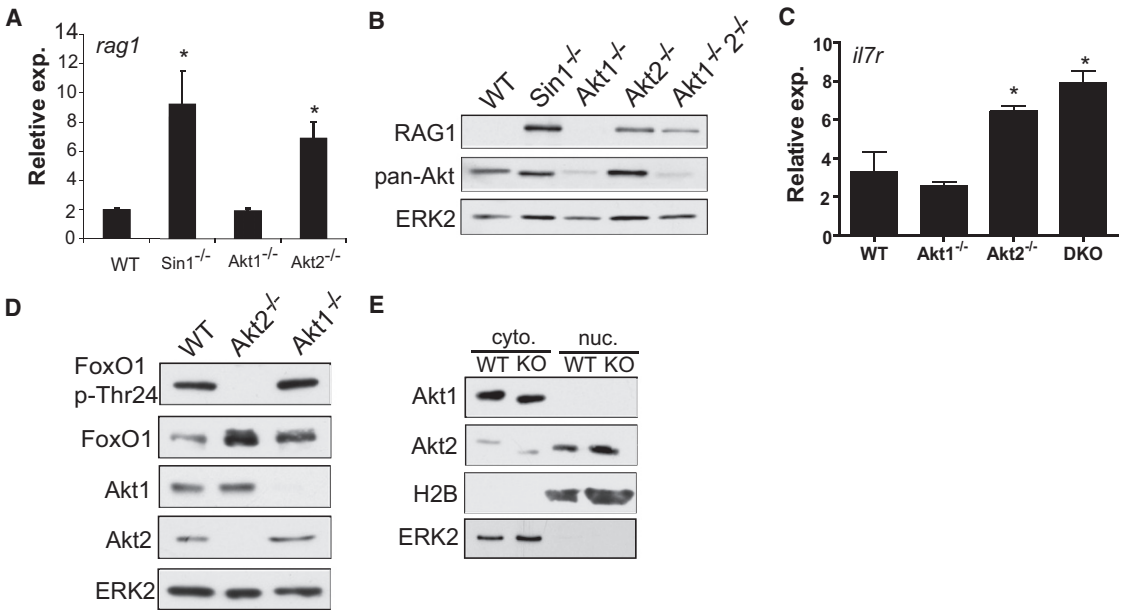

Figure 5. Akt2 Regulates Rag and IL-7R Expression in Pro-B Cells

(A) Wild-type (WT), *Sin1*$^{-/-}$, and Akt-deficient pro-B cells were cultured on OP9 cells with IL-7. *Rag1* mRNA levels were measured by qPCR and normalized to *GAPDH* mRNA. Samples were run in triplicate and are representative of two independent experiments (* = $p < 0.01$).

(B) RAG1 protein was measured in primary pro-B cells cultured on OP9 cells with IL-7. Total Akt expression was measured with a pan-Akt antibody. Samples were normalized to total cellular protein.

(C) *Il7r* mRNA expression in WT, *Akt1*$^{-/-}$, *Akt2*$^{-/-}$, or *Akt1*$^{-/-}$ *Akt2*$^{-/-}$ (DKO) pro-B cells cultured on OP9 cells with IL-7 was measured by quantitative RT-PCR and normalized to *GAPDH* mRNA. Samples were run in triplicate and are representative of two independent experiments (* = $p < 0.01$).

(D) FoxO1 phosphorylation was measured in wild-type, *Akt2*$^{-/-}$, or *Akt1*$^{-/-}$ pro-B cells. Samples were normalized to total cellular protein.

(E) Cytoplasmic and nuclear Akt1 and Akt2 was determined in *Sin1*$^{+/+}$ (WT) or *Sin1*$^{-/-}$ (KO) pro-B cells cultured on OP9 cells with IL-7. ERK2 and H2B proteins were used to verify the quality of the cytoplasmic and nuclear fractions. Three times more nuclear proteins (based on the total cell numbers) than cytosolic proteins were loaded in this assay to enhance the nuclear signals. Standard deviation is indicated by all error bars.

Sin1$^{-/-}$ *Akt2*$^{-/-}$, and *Akt1*$^{-/-}$/*Akt2*$^{-/-}$ pro-B cells but not wild-type or *Akt1*$^{-/-}$ pro-B cells (Figure 5B). We also examined IL-7R expression in Akt deficient pro-B cells and found that *il7r* expression was increased in *Akt2*$^{-/-}$ and *Akt1*$^{-/-}$/*Akt2*$^{-/-}$but not wild-type or *Akt1*$^{-/-}$ pro-B cells (Figure 5C). These results indicate that Akt2 is the principle mediator of Sin1/mTORC2 signaling, which suppresses *il7r* and *rag* expression in developing B cells.

To explore the mechanism though which Akt2 regulates *rag1* and *il7r* in B cells, we analyzed FoxO1 phosphorylation in *Akt1*$^{-/-}$ and *Akt2*$^{-/-}$ pro-B cells and found that the deletion of Akt2 but not Akt1 blocked FoxO1 Thr24 phosphorylation (Figure 5D). These results prompted us to examine how Akt2 might specifically regulate FoxO1 phosphorylation in B cells. We speculated that the differential subcellular localization of Akt1 and Akt2 proteins within pro-B cells may contribute to the specific regulation of FoxO1 by Akt2. Therefore, we fractionated cytosol and nuclear proteins from *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ pro-B cells and determined the distribution of Akt1 and Akt2 proteins in these subcellular fractions. We found that Akt1 was localized exclusively in the cytosol while Akt2 was localized in both cytosol and nucleus of pro-B cells (Figure 5E). These studies also revealed that the differential subcellular localization of Akt1 and Akt2 proteins in pro-B cells is not dependent on Sin1. Together, these data show that Akt2 specifically regulates FoxO1 phosphorylation in pro-B cells and suggests that the selective localization of Akt2 to the B cell nucleus contributes to the specific regulation of FoxO1 by Akt2.

Rapamycin Induces *rag* Expression in B Cells by Blocking mTORC2-Dependent Akt HM Phosphorylation

Prolonged rapamycin treatment is known to disrupt mTORC2 and inhibit Akt Ser473 phosphorylation in some cell types (Facchinetti et al., 2008; Sarbassov et al., 2006). Therefore we asked if rapamycin treatment may disrupt mTORC2, inhibit Akt HM phosphorylation, and induce *rag* gene expression in B cells. We treated wild-type Ab-MuLV pre-B cells with rapamycin for 5 hr or 25 hr and measured *rag* expression by quantitative RT-PCR. The acute rapamycin treatment (5 hr) did not alter *rag* expression, but prolonged rapamycin treatment (25 hr) substantially increased *rag1* and *rag2* mRNA levels (Figure 6A). In addition, rapamycin treatment also markedly increased the RAG1 protein level in Ab-MuLV pre-B cells (Figure 6B). We also observed that FoxO1 protein levels were increased after rapamycin treatment (Figure 6B). Furthermore, we observed that phosphorylation of Akt Ser473 was inhibited after 25 hr but not 5 hr of rapamycin treatment (Figure 6C). The overall Akt protein level was also reduced after 25 hr of rapamycin treatment. As expected, phosphorylation of the mTORC1 target 4E-BP1 was inhibited by rapamycin at 5 and 25 hr points

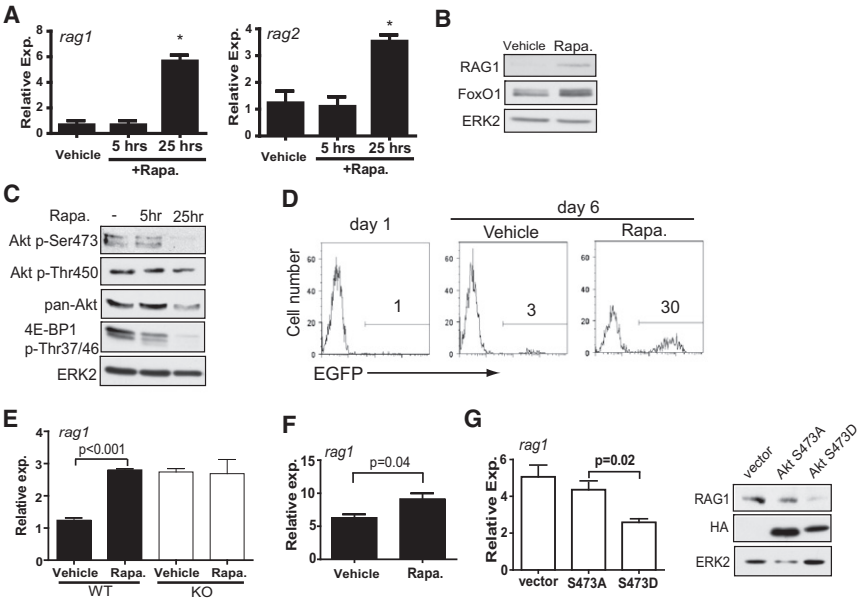

Figure 6. Rapamycin Induces RAG Gene Expression and Inhibits Akt Ser473 Phosphorylation in B Cells

(A) *Rag* mRNA levels were measured by qPCR in *Sin1*$^{+/-}$ Ab-MuLV pre-B cells treated with rapamycin for the indicated periods of time. *Rag1* and *rag2* expression was normalized to *GAPDH* mRNA. Samples were run in triplicate and are representative of two independent experiments (* = $p < 0.01$).

(B) *Sin1*$^{+/-}$ Ab-MuLV pre-B cells were cultured in the presence or absence of rapamycin for 25 hr and RAG1 or FoxO1 protein was measured. ERK2 is used as a loading control.

(C) *Sin1*$^{+/-}$ Ab-MuLV pre-B cells were vehicle or rapamycin treated for 5 or 25 hr. Cell lysates were normalized to total protein and immunoblotted as indicated.

(D) *Sin1*$^{+/-}$ Ab-MuLV pre-B cells were infected with a retrovirus containing an EGFP RAG recombinase reporter. The infected cells were continuously cultured in the presence (Rapa.) or absence (vehicle) of rapamycin for 6 days. The EGFP^{+} cells were determined by flow cytometry at 1 day and 6 days after infection. The plots are gated on infected cells (hCD4^{+} cells) and are representative of three independent experiments.

(E) *Sin1*$^{+/+}$ (WT) or *Sin1*$^{-/-}$ (KO) pro-B cells cultured on OP9 cells with IL-7 were treated with rapamycin or vehicle for 25 hr and *rag1* expression was measured by qPCR. Samples were normalized to *GAPDH* expression. Samples were run in triplicate and the data are representative of two independent experiments.

(F) Resting *Sin1*$^{+/-}$ splenic B cells were enriched by negative selection and cultured in vitro in the presence (Rapa) or absence (Vehicle) of rapamycin for 25 hr. *Rag1* expression was measured by qPCR and normalized to *GAPDH* expression. Samples were run in triplicate, and the data are representative of two independent experiments.

(G) *Sin1*$^{-/-}$ Ab-MuLV pre-B cells were infected with retrovirus expressing human Akt with a null HM mutation (S473A) or a phosphomimetic HM mutation (S473D). *Rag1* mRNA expression was measured by qPCR and normalized to *GAPDH*. RAG1 protein levels were measured by immunoblotting and the HA blotting verifies expression of the virally expressed Akt. Quantitative RT-PCR samples were run in triplicate and the data are representative of two independent experiments. Standard deviation is indicated by all error bars.

(Figure 6C). These data show that rapamycin induces *rag* gene expression in a manner that correlates with the loss of Akt Ser473 phosphorylation in B cells.

The increased *rag* expression in *Sin1*$^{+/-}$ pre-B cells following rapamycin treatment suggested that rapamycin may also increase V(D)J recombinase activity in B cells. We infected *Sin1*$^{+/-}$ Ab-MuLV pre-B cells with the EGFP RAG recombinase reporter and cultured these cells in the presence or absence of rapamycin. We observed that about 30% of the rapamycin treated, infected pre-B cells expressed EGFP while only 3% of the vehicle treated, infected cells expressed EGFP after 6 days (Figure 6D). These data show that rapamycin mimics the effect of Sin1 deficiency (Figure 4F) and promotes V(D)J recombinase activity in B cells.

Next, we asked if rapamycin induces *rag* expression in nontransformed B cells. We cultured *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells on OP9 cells with IL-7 in the presence or absence of rapamycin for 25 hr then measured *rag1* expression by quantitative RT-PCR. Rapamycin induced *rag1* expression in the *Sin1*$^{+/+}$ pro-B cells by approximately 3-fold, while rapamycin had no effect on *rag1* expression in *Sin1*$^{-/-}$pro-B cells (Figure 6E). Additionally, we also observed that rapamycin induced *il7r* mRNA levels by 2-fold in *Sin1*$^{+/+}$ pro-B cells (Figure S4). To further explore the effect of rapamycin on *rag* expression in B cells, we purified total splenic B cells from a *Sin1*$^{+/-}$ mouse and cultured these cells in vitro for 24 hr with or without rapamycin. We observed that *rag1* expression was increased in the rapamycin treated *Sin1*$^{+/-}$ splenic B cells when compared to vehicle treated B cells (Figure 6F). These data show that rapamycin treatment induces *il7r* expression in pro-B cells and *rag* expression in pro-B and splenic B cells.

Since Akt HM phosphorylation is dependent on mTORC2, we asked if Akt HM phosphorylation specifically mediates the Sin1/mTORC2-dependent inhibition of *rag* expression in B cells. We infected *Sin1*$^{-/-}$ Ab-MuLV pre-B cells with retrovirus expressing a human *Akt* cDNA with either a Ser473 to Ala null mutation or a phosphomimetic Ser473 to Asp mutation. Infected cells were sorted based on virally expressed GFP and *rag1* expression was measured by quantitative RT-PCR. Ectopic expression of the Ser473Ala Akt mutant failed to inhibit *rag1* expression in *Sin1*$^{-/-}$ pre-B cells. In contrast, expression of the Ser473Asp Akt mutant markedly reduced *rag1* expression in *Sin1*$^{-/-}$ pre-B cells (Figure 6G). Consistently, expression of the Ser473Asp Akt mutant but not the Ser473Ala Akt mutant suppressed RAG1 protein levels in Sin1$^{-/-}$ pre-B cells (Figure 6G). In addition, Ser473Asp Akt mutant also induced more FoxO1 phosphorylation than the Ser473Ala Akt mutant (data not shown). These data demonstrate that Akt HM site function is necessary and sufficient to complement Sin1 deficiency and suppress *rag* expression in developing B cells.

DISCUSSION

In this study we show that Sin1, an essential component of mTORC2, plays a critical role in B cell development. As illustrated in the model in Figure 7, Sin1/mTORC2 mediates PI3K-dependent signals (i.e., pre-BCR or BCR) to phosphorylate the hydrophobic motif (Ser473) of Akt2. PI3K-dependent PDK1 phosphorylates the T loop (Thr308) of Akt2 resulting in full Akt2 activation. Ser473 phosphorylation directs Akt2 activity toward its substrate FoxO1 Thr24 resulting in phosphorylation of FoxO1 thus suppressing the expression of FoxO1 target genes *il7r*, *rag1*, and *rag2* in developing B cells. Our study demonstrates the specific role of Sin1/mTORC2 and Akt2 as key regulators of *il7r* and *rag* gene expression in B cells.

Interestingly, Sin1 is not required for pro-B cell proliferation and survival (Figures S1A and S1B). Rather, *Sin1*$^{-/-}$ pro-B cells exhibit enhanced survival when cultured in the presence of IL-7. Our data indicates that this enhanced response to IL-7 is due to increased IL-7R expression on *Sin1*$^{-/-}$ pro-B cells. Our data also indicates that *Sin1* regulates pro-B cell to pre-B cell differentiation since we observe an accumulation of pro-B and a reduction of IgM^{+} immature B cells in the bone marrow of *Sin1*$^{-/-}$ chimeric mice and in *Sin1*$^{-/-}$ B cells differentiated in vitro on OP9 cells. Therefore, we propose that Sin1 mediates the PI3K-dependent pre-BCR signaling to suppress *rag* and *il7r* expression, which inhibits further IgH recombination.

It is of note that, mice lacking key structural components of pre-BCR such as Igμ, surrogate light chain genes λ5 and VpreB, and mice deficient in key pre-BCR signaling mediators such as Igα or Igβ all exhibit a B cell developmental block at the pro-B to pre-B transition (Herzog et al., 2009). The Sin1/mTORC2-Akt2-FoxO1 axis may also operate in a similar manner in transducing BCR signals in immature B cells following IgL recombination since the downstream mediators of pre-BCR and BCR signaling are conserved (Herzog et al., 2009). Therefore we predict that Sin1 deficiency may also perturb BCR signaling in immature and mature B cells. PI3K-dependent BCR signaling suppresses *rag* expression and inhibits IgL receptor editing in immature B cells (Verkoczy et al., 2007). We observe that *rag* gene expression is elevated in *Sin1*$^{-/-}$ immature B cells and that Igλ chain usage is increased in *Sin1*$^{-/-}$ B cells (Figures 4D and S3), suggesting that Sin1 mediates BCR-dependent PI3K signaling as well.

Sin1 deletion does not appear to impair Ig recombination. Rather, we observe increased *rag* expression and V(D)J recombinase activity in *Sin1*$^{-/-}$ B cells. Furthermore, the induction of Igκ germline transcription, which is an indicator of locus activation, is not impaired in *Sin1*$^{-/-}$ pre-B cells indicating that Sin1 is not required for the IgL locus accessibility (data not shown). In fact, the augmented V(D)J recombinase activity in developing *Sin1*$^{-/-}$ B cells may even promote IgL recombination since we observe an increase in the percentage of immature *Sin1*$^{-/-}$ B cells that express Igλ (Figures S3 and 4D).

Sin1 is an evolutionally conserved adaptor molecule and is essential for the integrity of mTORC2 in a diverse array of organisms and cell types (Jacinto et al., 2006; Yang et al., 2006). Sin1 deficiency blocks the phosphorylation of Akt at Ser473 and Thr450 in both primary pro-B and transformed pre-B cells without impairing Akt Thr308 phosphorylation. These results are consistent with our previous studies in nonimmune cells (Facchinetti et al., 2008; Jacinto et al., 2006). Our results also demonstrate that the mTORC2 is the sole kinase (PDK2) for Akt HM site phosphorylation in B cells.

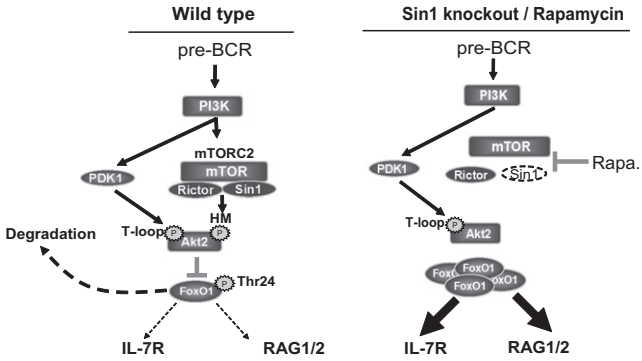

Figure 7. A Model Illustrating the Regulation of *il7r* and *rag* Expression by the Sin1/mTORC2-Akt2 Signaling Axis in Developing B Cells

PDK 1 and Sin1/mTORC2 mediate the PI3K signals to phosphorylate Akt2 at Thr308 and Ser473, respectively. Activated Akt2 then phosphorylates FoxO1, resulting in FoxO1 degradation and downregulation of *il7r* and *rag* gene expression. In the absence of Sin1 or during chronic rapamycin treatment, mTORC2 is disrupted and Akt2 is not fully activated, resulting in FoxO1 hypophosphorylation and accumulation; this leads to the augmented *il7r* and *rag* gene expression.

The expression of *rag1*, *rag2* and *il7r* is stringently regulated in developing B cells through a mechanism, which involves multiple signaling pathways. Our study reveals Sin1 as a key negative regulator of these genes in B cells whose activity is most likely mediated by FoxO1 since *rag1*, *rag2*, and *il7r* are direct targets of FoxO1 regulation (Amin and Schlissel, 2008; Dengler et al., 2008; Herzog et al., 2008). We present evidence showing that disruption of Sin1 or Akt2 impairs FoxO1 phosphorylation, which correlates with increased *rag1/2* and *il7r* expression. Furthermore, we demonstrate the functional importance of Akt HM phosphorylation in regulating FoxO1 activity by showing that ectopic expression of the phosphomimetic Ser473Asp Akt mutant but not the phosphorylation null Ser473Ala Akt mutant inhibits *rag* expression in *Sin1*$^{-/-}$ pre-B cells. Interestingly, we observed that the relative amount of nuclear localized FoxO1 protein is similar in *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ pro-B cells (data not shown). These data suggest that the relative abundance of nuclear FoxO1 protein is not tightly correlated with FoxO1 transcriptional activity and that regulation of FoxO1 localization in B cells is Sin1/mTORC2 independent.

Although Sin1/mTORC2 regulates both Akt1 and Akt2 HM and TM site phosphorylation, Akt1 and Akt2 appear to be differentially utilized by developing B cells with respect to the regulation of *rag1*, *rag2*, and *il7r*. We were particularly surprised to find that Akt1 does not regulate FoxO1 phosphorylation or *rag1*, *rag2*, and *il7r* expression since Akt1 is the most abundantly expressed Akt isoform in developing B cells (Figure 5B). We show that Akt2, but not Akt1, is selectively localized to the nucleus of B cells. Based on these data we propose that nuclear localized Akt2 is responsible for phosphorylating FoxO1. Exactly how this selective,

isoform specific, cellular distribution of Akt proteins is achieved and regulated in B cells remains to be elucidated. However, we observe that Akt2 is nuclear localized in *Sin1*$^{-/-}$ pro-B cells suggesting that Akt HM and TM phosphorylation are not required for Akt2 import into the nucleus. These results indicate that Akt2 HM phosphorylation may be required to specifically facilitate interaction of Akt2 with FoxO1 or promote the Akt2-dependent phosphorylation of FoxO1 at Thr24.

V(D)J recombination is normally suppressed in proliferating B cells by coupling RAG2 protein degradation with mitosis (Li et al., 1996). Cell-cycle-dependent regulation of V(D)J recombination helps to ensure that aberrant recombination products are not generated during the cell cycle, which may result in mutations that promote tumor formation. Our data reveals that Sin1/mTORC2 signaling provides an additional level of protection against abnormal V(D)J recombinase activity by suppressing expression of *rag1* and *rag2* in proliferating B cells. Surprisingly, we observed substantial V(D)J recombinase activity in *Sin1*$^{-/-}$ Ab-MuLV pre-B leukemia cells that were actively proliferating (Figure 4F). These findings raise the possibility that mTOR inhibitors, which disrupt mTORC2 function and induce *rag* expression, may promote genome instability in B cells by promoting aberrant V(D)J recombinase activity. This is a very important point in light of our finding that rapamycin increases *rag* expression in mature B cells. Our data argues that the *rag* locus is not irreversibly silenced upon B cell maturation and suggests that mTORC2-dependent signaling actively suppresses *rag* expression in mature B cells. This has significant implications with regard to the mechanisms that lead to genome instability and B cell tumors. Perturbations of mTORC2 signaling that induce *rag* expression in immature or mature B cells may increase the likelihood of coexpression of *rag* with activation-induced cytidine deaminase (AID), a circumstance that has been strongly implicated in the generation of chromosomal translocations (Tsai et al., 2008; Wang et al., 2008, 2009). Future studies will elucidate the role of mTORC2 in promoting B cell genome stability and determine if pharmacologic mTOR inhibition increases the likelihood of generating B cell tumors.

EXPERIMENTAL PROCEDURES

A detailed explanation of the experimental methods can be found in the Supplemental Experimental Procedures.

Mice

Sin1 knockout mice were described previously (Jacinto et al., 2006). Akt1 and Akt2 knockout mice were described previously (Di Lorenzo et al., 2009). CD45.1^{+} congenic (B6.SJL-*Ptprc*^{a}) mice were purchased from The Jackson Laboratory and used as recipients for the fetal liver hematopoietic cell transfers. Mice receiving fetal liver cell transplants were irradiated with 700–900 cGy. All mice were housed in the animal facilities at Yale University, and all animal procedures were approved by the Yale Institutional Animal Care and Use Committee.

B Cell Cultures

Pro-B cells were derived from paired *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ littermate E12.5 embryos. *Akt2*$^{-/-}$ and *Akt1*$^{-/-}$/*Akt2*$^{-/-}$ pro-B cells were derived from E13.5 embryos, and *Akt1*$^{-/-}$ pro-B cells were derived from the bone marrow of a 6 week old Akt1$^{-/-}$ mouse. All pro-B cells were cultured on OP9 stromal cells in medium supplemented with recombinant mIL-7 (PeproTech). Abelson murine leukemia virus transformed pre-B cells were generated by infecting cultured pro-B cells with viral supernatant (kindly provided by Dr. Yuan Zhuang, Duke University).

Inhibitors

Imatinib (10 mM, LC Laboratories) stocks were prepared in sterile water and used at a final concentration of 10 μM. Rapamycin (LC Laboratories) was prepared as a 10 μM stock in ethanol and used at a final concentration of 10 nM in all studies unless otherwise indicated.

Flow Cytometry

Single cell suspensions were stained in cold FACS buffer (1× PBS pH7.4 + 2% FBS) with the appropriate fluorophore or biotin conjugated antibodies for 15 min on ice. For biotin conjugated antibodies, cells were washed with FACS buffer and incubated with the appropriate streptavidin conjugated fluorophores for 15 min on ice. Anti-IgM μ chain specific F(ab′)$_2$ fragment (Jackson ImmunoResearch) was used for intracellular IgH staining. All cells were washed and resuspended in FACS buffer for analysis with a FACSCalibur (BD).

Quantitative RT-PCR

Cells were lysed in TRIzol (Invitrogen); total RNA was purified by isopropanol precipitation. Total RNA was treated with RNase free DNase I (Sigma) and reverse transcribed with Super-Script II reverse transcriptase (Invitrogen) using random primers. Quantitative RT-PCR was performed with an iQ5 multicolor RT-PCR detection system (Bio-Rad) using the Power SYBR green PCR master mix kit (Applied Biosystems).

V(D)J Recombinase Activity Reporter

Ab-MuLV pre-B cells were infected with the retroviral vector pMX-RSS-GFP/IRES-hCD4, which contains a RAG recombinase activity reporter cassette and an IRES-hCD4 expressing cassette (Liang et al., 2002). The infected cells were gated for human CD4 expression and analyzed for the EGFP-positive cells at various times following infection.

Immunoblotting and Antibodies

Cells were washed 2× with ice cold 1× PBS and lysed in cold RIPA buffer with freshly added protease and phosphatase inhibitors. Total cell lysates were resolved by SDS-PAGE and blotted with the following antibodies: anti-Akt p-Thr308, anti-Akt p-Ser473 (587F11), anti-panAkt (11E7), anti-Akt2 (D6G4), anti-PKCα/βII p-Thr641/641, anti-Foxo1/3a p-Thr23/32, anti-Foxo1 (C29H4), anti-4E-BP1 p-Thr37/36 from Cell Signaling, anti-Akt1 (E45W), anti-H2B (EP819Y) (Epitomics), anti-Sin1 (K87) (Jacinto et al., 2006), anti-Rictor (Bethyl Inc.), anti-mTOR (N5D11) (IBL), anti-RAG1 (35.2) (Leu and Schatz, 1995), ERK2 (381A10) (Invitrogen). Densitometry analysis was performed with a BioRad Molecular Imager Gel Doc XR system and Quantity One software (BioRad). Immunoprecipitation of mTORC2 was performed as previously described (Facchinetti et al., 2008).

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.molcel.2010.07.031.

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A.S.L. designed and performed experiments, analyzed data, and wrote the paper; D.L and V.F. performed experiments; A.D.L., W.C.S., and D.G.S. provided key experimental reagents; D.G.S. assisted in experiment design; and B.S. designed experiments, analyzed data, and wrote the paper.

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