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Ras orchestrates cell cycle exit and light chain recombination during early B cell development

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Abstract

Signals through the pre-B cell antigen receptor (pre-BCR) and IL-7 receptor (IL-7R) coordinate pre-B cell expansion with subsequent *Ig*κ recombination. While many downstream effectors of each receptor are known, how they integrate to mediate development has remained unclear. Herein, we report that pre-BCR mediated activation of the Ras/MEK/ERK signaling pathway silences *Ccnd3* transcription and coordinates cell cycle exit with the induction of E2A and the initiation of *Ig*κ recombination. These activities are opposed by IL-7R mediated STAT 5 activation which promotes *Ccnd3* expression and concomitantly inhibits *Ig*κ transcription by binding to $E_{\kappa i}$ and inhibiting E2A recruitment. Our data reveal how pre-BCR signaling poises pre-B cells to undergo differentiation upon escape from IL-7R signaling.

INTRODUCTION

Following commitment to the B cell lineage, pro-B cells initiate recombination of the immunoglobulin heavy chain variable (V) to the diversity (D) and joining (J) gene segments. In-frame rearrangements allow the expression of Igμ which then assembles with Igα/Igβ, and the surrogate light chain complex (SLC, λ5 and VpreB), to form the pre-B cell antigen receptor (pre-BCR)1›2. Initial signals transmitted through the pre-BCR synergize with those elicited through the IL-7 receptor (IL-7R) to expand the B cell progenitor pool 3−5.

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AUTHOR CONTRIBUTIONS

The majority of the experiments in this manuscript were designed, performed, interpreted and readied into figures by Malay Mandal. He also assisted in preparing the initial manuscript. Sarah Powers assisted in the design and analysis of many of the experiments. Kyoko Ochiai assisted with the chromatin immunoprecipitations. Katia Georgopoulos provided the Aiolos specific reagents, scientific advice and assisted in the preparation of the manuscript. Barbara Kee provided E2A specific reagents, contributed to the design of some experiments, provided scientific advice and edited the manuscript. Harinder Singh assisted in establishing many of the experimental systems necessary for this project. He also provided substantial scientific input and edited the final manuscript. Marcus Clark oversaw the entire project and prepared the final manuscript.

For subsequent development, pre-B cells must cease dividing and then initiate light chain recombination, primarily at the *Igκ* locus[3,4]. These two fundamental processes are, by necessity, sequential as concurrent replication and the introduction of double-strand breaks during recombination would compromise genomic integrity[6]. While it is clear that the pre-BCR and the IL-7R control proliferation and recombination, it is not known how receptor initiated signals integrate to affect this developmental transition. It has been hypothesized that proliferating pre-B cells exit cell cycle upon termination of SLC expression[7,8]. In this model, it is the absence of pre-BCR signals that mediate cell cycle exit. However, recent experiments in which SLC was constitutively expressed indicate that down-regulation of the pre-BCR is not required to either limit proliferation or to initiate *Igκ* rearrangement[9]. Furthermore, loss of downstream components of the pre-BCR signaling cascade, including SLP-65 and PLCγ2, arrests development at the cycling pre-BCR$^+$ stage[10–12]. These observations indicate that the signaling mechanisms limiting proliferation are intrinsic to the pre-BCR.

Cessation of cell cycle in pre-B cells is not sufficient to induce *Igκ* recombination[13]. Rather, *Igκ* recombination appears to be directly regulated by the pre-BCR. Transgenic Igm expression increases *Igκ* locus accessibility in recombinase activating gene 2 (*Rag2*$^{-/-}$) pro-B cells[14] while targeted mutation of some pre-BCR signaling cascade components both enhances proliferation and attenuates *Igκ* recombination[11,15]. These latter observations implicate common proximal signaling molecules in *Igκ* recombination and cell cycle exit.

The signaling intermediate Ras has been implicated in B lymphopoiesis. Expression of dominant negative (DN) Ras results in a developmental block at the pro-B cell stage, before expression of the pre-BCR[16]. In contrast, expression of constitutively active (CA) Ras in *Rag1* deficient mice induces a unique population of cells expressing markers of later B cell development[17]. These latter results indicate that downstream of the pre-BCR, Ras is an important mediator of developmental progression. Consistent with this conclusion, expression of CA-Ras in $J_H^{-/-}$ mice induces extensive *Igκ* rearrangement[18]. The molecular mechanisms by which Ras contributes to *Igκ* rearrangement are not known. Furthermore, the relationships between Ras mediated *Igκ* recombination and cell cycle regulation have not been explored.

Downstream of the pre-BCR, the interferon regulatory factor family members, IRF4 and IRF8, are required *in vivo* for initiating light chain recombination, suppressing SLC and exiting cell cycle[19,20]. Culture of *Irf4*$^{-/-}$*Irf8*$^{-/-}$ bone marrow with IL-7 yields populations of actively cycling pre-BCR$^+$ cells while reintroduction of IRF4, in the presence of IL-7, silences SLC[20] and induces *Igκ* recombination[21,22]. These and other observations[23] demonstrate the importance of IRF4 in the generation of immature B cells.

While *in vivo Irf4*$^{-/-}$*Irf8*$^{-/-}$ mice manifest a complete block in B lymphopoiesis, attenuation of IL-7R signaling in *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cell cultures induces cell cycle exit and *Igκ* recombination[22]. These observations suggest the existence of two molecular pathways that function synergistically to activate *Igκ* rearrangement: one that is independent of IRF-4 and requires attenuation of IL-7R signaling and another dependent on pre-BCR induced IRF-4 expression. The regulation of *Igκ* recombination by distinct signaling pathways is consistent with the known composite regulation of *Igκ* transcription. IRF-4 augments *Igκ* transcription through the *Igκ* 3' enhancer ($E_{\kappa 3'}$) while the IL-7R represses *Igκ* trancription through the intronic *Igκ* enhancer ($E_{\kappa i}$)[22].

In addition to directly regulating *Igκ* recombination, IRF-4 upregulates CXCR4 and promotes CXCL2 responsiveness of pre-B cells[22]. Given that there are discrete bone marrow niches of IL-7 and CXCL12 expression[24] the *in vivo* phenotype of *Irf4*$^{-/-}$*Irf8*$^{-/-}$ B

cell progenitors may thus reflect, in part, an inability to migrate into IL-7 deficient microenvironments[22].

Several other transcription factors have been implicated in the regulation of the *Igκ* locus[25]; however, expression of the transcription factor E2A with RAG1 and RAG2 is sufficient to induce *Igκ* recombination in a non-lymphoid cell line [26]. Furthermore, gene-targeting studies have demonstrated that mutation of the E2A binding motifs in the $E_{\kappa i}$ reduces *Igκ* rearrangement similarly as ablation of the entire $E_{\kappa i}$[27]. These, and other studies[23,28], demonstrate that E2A is an important direct regulator of *Igκ* transcription. The activity of E2A proteins, and the regulation of E2A dependent developmental events, is modulated by expression of the inhibitors of DNA binding proteins, Id1–3[29].

The mechanisms regulating *Igκ* recombination have been intensely studied. In contrast, the important regulators controlling cell cycle progression in pre-B cells have only recently been identified[13,30]. Cyclin D3 expression is upregulated in pre-B cells and the pre-B cell proliferative expansion is ablated in *Ccnd3*$^{-/-}$ mice[30]. Furthermore, exit from cell cycle, and the initiation of *Igκ* recombination, is associated with termination of *Ccnd3* transcription[30]. Therefore, to understand the relationships between the signaling elements controlling cell cycle exit and *Igκ* recombination, we first sought to identify the negative regulators of *Ccnd3* transcription and relate these to those controlling E2A.

Herein, we demonstrate that pre-BCR mediated Ras/MEK/ERK activation couples cell cycle exit to *Igκ* recombination. Downstream of ERK, Aiolos silences *Ccnd3* while reciprocal regulation of E2A and Id3 initiates *Igκ* transcription. The IL-7R antagonizes *Igκ* transcription through STAT 5, which binds to the *Igκ* intronic enhancer and prevents E2A recruitment. These findings identify a central signaling pathway controlling pre-BCR development and provide a molecular framework for understanding how the balance between pre-BCR and IL-7R signaling determines cell fate.

RESULTS

Ras-MEK activation is required for cell cycle exit and *Igκ* recombination in pre-B cells

To better understand how proliferation is regulated during B lymphopoiesis, we sought to identify the specific signaling pathways that suppressed *Ccnd3* transcription in pre-B cells. For these initial studies, we used cultured *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells as our model of differentiation. In the presence of IL-7, these pre-BCR^{+} cells continually proliferate. However, upon attenuation of IL-7 signaling they exit cell cycle and initiate *Igκ* recombination[19,22].

In complete media alone, withdrawal of IL-7 was associated with diminished abundance of *Ccnd3*, *Ccnd2* and *Ccne* encoding mRNAs (Fig. 1a) as well as the corresponding protein products (Fig. 1b). Initial pharmacological inhibitor studies failed to identify any negative regulators of *Ccnd3* transcription[30]. However, subsequent studies demonstrated that inhibiting either MEK or ERK abrogated IL-7 withdrawal-induced cyclin downregulation (Fig. 1a, 1b). The observed modulation of cyclin E was proportional to that observed for the D-type cyclins, consistent with cyclin D mediated regulation of cyclin E[31].

We next examined if the MEK/ERK pathway could regulate processes necessary for light chain recombination. Transcription of *Rag1*, *Rag2* (Fig. 1c) and *Igκ* germline (Fig. 1d) was induced upon IL-7 withdrawal. Inhibition of either MEK or ERK attenuated both *Rag* and *Igκ* germline transcription following IL-7 withdrawal (Fig. 1c, 1d). MEK inhibition for 24 hours did not affect surface expression of the pre-BCR (data not shown). These data

suggested that signaling through the MEK/ERK pathway directly impacts both cell cycle and the initiation of *Ig*κ recombination.

To determine if the above observations were relevant to normal B lymphopoiesis, we examined ERK activation in different B cell progenitor populations. Pro-B ($B220^+CD43^+IgM^-$), large pre-B ($B220^+CD43^{low/-}IgM^-$) and small pre-B cells were isolated from wild-type (WT) bone marrow by flow cytometry, permeabilized and stained with anti-phospho-ERK antibodies. As can be seen in Fig. 1e, ERK activation was observed in all three populations with the highest levels detected in large pre-B cells and the lowest in pro-B cells.

The pattern of ERK activation paralleled that of pre-BCR expression which is highest in large pre-B cells and is then attenuated upon transition to the small pre-B cell stage[9]. To determine if expression of the pre-BCR augmented ERK activation, cultured *Rag2*$^{-/-}$ pro-B cells were infected with control retrovirus (MIGR1-IRES-GFP) or retrovirus encoding Igμ[32] and GFP^+ cells were assessed by flow cytometry. As can be seen in Fig. 1f, expression of Igμ led to the surface expression of a pre-BCR and this was associated with increased ERK activation.

To assess the function of MEK/ERK activation, large and small pre-B cells were isolated by fluorescence-activated cell sorting (FACS), treated for six hours in short-term cultures with MEK inhibitor and then assayed for *Ccnd3* and germline *Ig*κ transcription (Fig. 1g and 1h). As expected, large pre-B cells had relatively high levels of *Ccnd3* transcripts and low levels of *Ig*κ germline transcripts compared to small pre-B cells. In both cell populations, inhibition of MEK suppressed the abundance of *Ig*κ germline transcripts and augmented those of *Ccnd3*. These results validate our initial observations in cultured *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells and indicate that pre-BCR mediated MEK/ERK activation plays a central role in coordinating *Ccnd3* suppression with the induction of *Ig*κ germline transcription.

To further explore the contributions of the MEK/ERK signaling pathway to B lymphopoiesis, cultured *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells were infected with control retrovirus or retrovirus encoding either a dominant negative (DN) mutant of Ras (DN-Ras, N^{17}-H-Ras) or MEK (DN-MEK, MKK1-E^8-K^{97}M). GFP expressing cells were isolated by FACS, expanded in IL-7, and then cultured in either the presence or absence of IL-7. Both pharmacological inhibition and expression of DN-Ras and DN-MEK attenuated ERK activation (Fig. 2a). Interestingly, IL-7 withdrawal did not significantly modulate ERK activation in *Irf4*$^{-/-}$*Irf8*$^{-/-}$ cells and ERK phosphorylation was relatively low in *Rag2*$^{-/-}$ pro-B cells irrespective of IL-7. These observations are consistent with the above results (Fig. 1e, f), and the findings of others, indicating that ERK activation primarily lies downstream of the pre-BCR[33,34,35].

Ectopic expression of either DN-Ras or DN-MEK increased the level of *Ccnd3* mRNA in the presence of IL-7 and prevented the usual decrease associated with IL-7 withdrawal (Fig. 2b). Parallel changes in cyclin D3 protein expression were observed (Fig. 2c). Ectopic expression of both DN-Ras and DN-MEK similarly increased cyclin D2 and cyclin E expression (Supplementary Fig. 1a–1c). Ectopic expression of DN-Ras or DN-MEK did not significantly alter expression of *p21Cip1* (Supplementary Fig. 1d) and only partially blunted the induction of *p27Kip1* observed following IL-7 withdrawal (Supplementary Fig. 1e).

We next examined the role of Ras and MEK activation in cell cycle exit following IL-7 withdrawal. *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells expressing either DN-Ras or DN-MEK were cultured in the presence or absence of IL-7 for up to 48 hours. Cell aliquots were permeabilized, stained with propidium iodide and analyzed by flow cytometry. In the presence of IL-7, both DN-Ras and DN-MEK modestly increased the fraction of cells in S/G_2M (Fig. 2d).

However, expression of either DN-Ras or DN-MEK significantly blunted the cell cycle exit associated with IL-7 withdrawal; 48 hours after IL-7 withdrawal when only 7% of control transfected cells were in S/G_2M, 21% of those expressing DN-Ras and 18% of those expressing DN-MEK were in cell cycle. These data indicate that escape from IL-7 is not sufficient to terminate pre-B cell proliferation. In addition, activation through the Ras/MEK pathway is required to suppress *Ccnd3* transcription and exit cell cycle.

We next examined the role of Ras/MEK/ERK activation in regulating the mechanisms of *Ig*κ recombination. As demonstrated in Fig. 3, withdrawal of IL-7 from cultured *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells induced the expression of *Rag1* and *Rag2* (Fig. 3a and 3b) and enhanced *Ig*κ germline transcription (Fig. 3c). These responses were significantly attenuated by expression of either DN-Ras or DN-MEK.

To determine if the observed regulation of *Rag* and *Ig*κ germline transcription affected *Ig*κ recombination, genomic DNA was isolated from aliquots of cultured *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells expressing MIGR1, DN-Ras or DN-MEK. Samples were then subjected to PCR with oligonucleotide primers complementary to Vκ and $J\kappa_{1-5}$ and the products visualized as described[22]. The results of a typical experiment are provided in Fig 3d. Fig. 3e provides a quantitative comparison of the relative band intensities corresponding to each V-$J\kappa_{1-5}$ recombination from three independent experiments. As can be seen, expression of either DN-Ras or DN-MEK significantly diminished *Ig*κ recombination. From these observations, we conclude that the Ras/MEK/ERK signaling pathway coordinates both cell cycle exit and *Ig*κ recombination in pre-B cells.

Ras-MEK mediated cyclin D3 suppression, and cell cycle exit, are dependent on Aiolos

We next asked if the Ras/MEK/ERK pathway suppressed proliferation and induced *Ig*κ recombination through the same or different downstream signaling pathways. Aiolos is a lymphoid restricted transcription factor that is specifically upregulated in pre-B cells[36] by pre-BCR dependent signaling pathways[37,38]. Furthermore, pre-B cell proliferation is increased in *Aiolos*$^{-/-}$ mice[39]. These findings prompted us to examine if Aiolos was playing a role in Ras/MEK mediated cell cycle exit.

We first characterized the expression of Aiolos, and the family member Ikaros[40], in FACS isolated pro-B ($CD19^+CD43^{high}IgM^-$) and pre-B ($CD19^+CD43^{low/-}IgM^-$) cells from wild-type (WT) bone marrow. As expected[37], *Aiolos* expression was increased in pre-B cells relative to pro-B cells, whereas the expression of *Ikaros* was similar in both populations (Fig. 4a). We next determined if the Ras/MEK/ERK signaling pathway could modulate *Aiolos* expression. *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells ectopically expressing either DN-Ras or DN-MEK were cultured in the presence or absence of IL-7 and then assayed for the expression of *Aiolos* (Fig. 4b) and *Ikaros* (Fig. 4c). As demonstrated, blocking the Ras/MEK pathway attenuated *Aiolos* expression but had no significant effect on *Ikaros* expression.

To determine if Aiolos could regulate *Ccnd3*, retroviral infection was used to establish cultured *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cell populations over-expressing murine Aiolos. In both the presence and absence of IL-7, ectopic expression of Aiolos downregulated *Ccnd3* mRNA (Fig. 4d). Furthermore, irrespective of IL-7, this was associated with a marked decrease of cells in S/G2M (Fig. 4e). Expression of Aiolos suppressed *Ccnd2* and *Ccne* expression but had no significant effect on the expression of *p21Cip1* or *p27Kip1* (Supplementary Figs. 2a–2b). Furthermore, *Aiolos* did not modulate *Rag1* or *Rag2* expression or enhance *Ig*κ transcription (Supplementary Fig. 2c and 2d). These data suggest that, in the context of pre-B cell differentiation, Aiolos functions downstream of Ras/MEK to specifically suppress *Ccnd3* expression and facilitate cell cycle exit.

To examine if Aiolos was required for Ras-mediated cyclin D3 downregulation, pre-B cells ($CD19^+CD43^{low}IgM^-$) were isolated by FACS from WT and *Aiolos*$^{-/-}$ bone marrow and cultured cells were infected with retrovirus encoding CA-Ras. GFP expressing cells were then isolated by FACS and cultured in IL-7. Expression of CA-Ras induced Aiolos expression in WT pre-B cells but not *Aiolos*$^{-/-}$ pre-B cells (Fig. 5a). As expected, CA-Ras also downregulated *Ccnd3* in WT cells. In contrast, in *Aiolos*$^{-/-}$ pre-B cells *Ccnd3* mRNA was elevated and could not be suppressed by CA-Ras (Fig. 5b). Both *Ccnd2* (Supplementary Fig. 3a) and *Ccne* (Supplementary Fig 3b) demonstrated a similar Aiolos dependent regulation by Ras. Cell cycle analysis revealed that ectopic expression of CA-Ras attenuated the fraction of cells in S/G_2M in WT but not *Aiolos*$^{-/-}$ pre-B cells (Fig 5c). These data indicate that the induction of Aiolos by Ras/MEK/ERK mediates cell cycle exit by inhibiting *Ccnd3* expression.

The magnitude of Ras-mediated *Aiolos* induction in cultured WT pre-B cells (Fig. 5a) and in *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells (Fig. 4b) was similar to the difference in expression observed between WT pro- and pre-B cells (Fig. 4a). However, overall Aiolos expression levels were lower in *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells than WT pre-B cells. This later observation is consistent with previous findings that IRF4 contributes to *Aiolos* induction [20]. However, our data indicate that sufficient Aiolos can be induced in the absence of IRF4 to mediate cell cycle exit.

Regulation of E2A and Id3 by the Ras/MEK/ERK signaling pathway enhances *Igκ* transcription

We next sought to identify which signaling intermediates downstream of Ras/MEK/ERK regulated *Igκ* recombination. We focused on *Igκ* transcription because it was almost entirely dependent upon Ras activation (Fig. 3c).

Igκ transcription is dependent upon E2A[23,26,27], which in turn is negatively regulated by the Id proteins (Id1–3)[41–43]. Therefore, we examined if Ras/MEK/ERK activation could play a role in the coordinate regulation of E2A and Id3 during B lymphopoiesis.

Irf4$^{-/-}$*Irf8*$^{-/-}$ untransfected pre-B cells or cells expressing DN-Ras or DN-MEK were cultured in the presence or absence of IL-7 and assayed for expression of the Id proteins. As can be seen in Fig. 6a, inhibiting the Ras/MEK pathway significantly augmented the expression of *Id3*. In contrast, DN-Ras or DN-MEK had no effect on *Id1* expression (Supplementary Fig. 4a) and moderately enhanced *Id2* expression (Supplementary Fig. 4b). Immunoblotting of lysates from *Irf4*$^{-/-}$*Irf8*$^{-/-}$ cells expressing either DN-Ras or DN-MEK confirmed that activation of the Ras/MEK/ERK pathway suppressed *Id3* expression (Fig 6b). Immunblotting of these same lysates also demonstrated that Ras/MEK induced E47 expression. Expression of both Id3 and E47 was not significantly affected by IL-7.

Quantitation of triplicate immunoblots indicated that inhibiting Ras or MEK led to an average 3.3-fold enhancement of Id3 expression (Fig. 6c) and a 2.9-fold decrease in E47 expression (Fig. 6d) irrespective of IL-7. While each observed change was moderate, the predicted change in the E47/Id3 ratio, and therefore of free E47, was substantial (9 to 10 fold). Therefore, we performed electrophoretic mobility shift assays (EMSAs) using nuclear extracts from *Irf4*$^{-/-}$*Irf8*$^{-/-}$ cells and an oligonucleotide probe corresponding to E1 box of $E_{\kappa i}$ (Fig. 6d). In either the presence or absence of IL-7, an E47 containing complex was detected that bound the WT, but not mutant, labeled oligonucleotide probe. This complex was attenuated by addition of either anti-E47 antibodies or by excess unlabelled WT oligonucleotides. In contrast, the complex was not diminished by either addition of isotype-matched control antibodies or by unlabelled oligonucleotides containing mutations in the E2A binding motif. Inhibition of MEK greatly attenuated the formation of detectable E47

containing binding complexes. In contrast, inhibition of MEK had no detectable effect on the binding activity of STAT 5 to a known binding site in *FcγRI*[44]. Consistent with previous observations, IL-7 had no significant effect on nuclear E2A binding activity[22] (Supplementary Fig. 5). These data indicate that MEK activation enhances E2A binding activity by coordinately regulating the expression of E2A and Id3.

To examine the potential importance of Ras/MEK/ERK regulation of Id3, *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells expressing a retrovirally encoded estrogen receptor (ER)-Id3 fusion protein were cultured with or without IL-7 in the presence or absence of 4-OH-tamoxifen. Although the expression of ER-Id3 and induction with tamoxifen had no significant effect on *Rag1* and *Rag2* expression (Fig. 7a), Id3 ablated detectable *Igκ* germline transcription (Fig. 7b). This was associated with complete suppression of detectable *Vκ* to *Jκ* recombination (Fig. 7c). Id3 induction had no effect on expression of the D- and E-type cyclins (Supplementary Fig. 4c) or on the cell cycle inhibitors *p21Cip1* and *p27Kip1* (Supplementary Fig. 4d). These data implicate Id3 as a specific regulator of *Igκ* transcription in pre-B cells.

To determine if Id3 was required to suppress *Igκ* transcription *in vivo*, WT or *Id3*$^{-/-}$ pre-B cells were isolated by FACS, cultured in IL-7 and then subjected to IL-7 withdrawal. As can be seen in Fig. 7d, *Igκ* transcription was significantly enhanced in *Id3*$^{-/-}$ pre-B cells in both the presence and absence of IL-7. Consistent with results obtained from ectopic Id3 expression, the expression of *Rag1* and *Rag2* was not altered in *Id3*$^{-/-}$ pre-B cells as compared to WT cells (Fig. 7e); furthermore, *Ccnd2*, *Ccnd3* and *Ccne* expression was comparable between WT and *Id3*$^{-/-}$ cells (Supplementary Fig. 6). To determine if Id3 regulates *Igκ* recombination *in vivo*, large and small pre-B cells from WT and *Id3*$^{-/-}$ mice were isolated, stained with anti-Igκ antibodies and analyzed by flow cytometry. As demonstrated in Fig. 7f, a greater fraction of *Id3*$^{-/-}$ large and small pre-B cells expressed Igκ on their cell surface, consistent with *Igκ* dysregulation. These data indicate that Ras/MEK/ERK enhances *Igκ* transcription through the coordinated regulation of E2A and Id3.

STAT 5 regulates accessibility of $E_{κi}$ to E2A

For Ras/MEK/ERK activation to efficiently induce cell cycle exit and *Igκ* recombination, pre-B cells must escape the effects of IL-7. Our data predict that IL-7 inhibits differentiation by opposing the Ras/MEK/ERK signaling pathway. However, IL-7 did not significantly alter Aiolos expression (Fig. 4b) or E2A binding activity[22] (Supplementary Fig. 5). This suggests that IL-7 modulates the activities of Ras/MEK/ERK downstream of Aiolos and E2A. To examine this possibility, we first sought to identify which IL-7R dependent signaling pathways enhance *Ccnd3* transcription and inhibit *Igκ* transcription.

Downstream of the IL-7R, there are two primary signaling pathways: JAK3/STAT 5 and PI-3 kinase/Akt[45]. Therefore, we examined if retroviral mediated expression of either CA-STAT 5B[46,47] or CA-Akt[48] could supplant the biological activities of IL-7 in cultures of *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells. As demonstrated in Fig. 8a, CA-STAT 5B enhanced *Ccnd3* expression in IL-7 treated cells and prevented the attenuation associated with IL-7 withdrawal. CA-Akt did not significantly affect *Ccnd3* expression. CA-STAT 5B also repressed *Igκ* germline transcription in cells cultured with or without IL-7 (Fig. 8b). In contrast, CA-Akt, and not CA-STAT 5B, repressed *Rag1* and *Rag2* induction following IL-7 withdrawal (Fig. 8c). From these data, we conclude that that STAT 5 is the main downstream signaling effector of the IL-7R regulating *Ccnd3* and *Igκ* transcription [47].

IL-7-mediated *Igκ* repression is dependent upon the $E_{κi}$. Furthermore, repression is associated with inhibition of E2A binding to $E_{κi}$ *in vivo*[22]. Therefore, we next examined if STAT 5 could modulate E2A binding to $E_{κi}$. We first determined if STAT 5 bound directly to $E_{κi}$ *in vivo*. Aliquots of *Irf4*$^{-/-}$*Irf8*$^{-/-}$ cells, cultured in the presence or absence of IL-7,

were subjected to quantitative chromatin immunoprecipitation (ChIP) assays with anti-STAT 5B antibodies. As demonstrated in Fig. 8d, STAT 5B bound to $E_{\kappa i}$ in an IL-7 dependent manner. Binding was robust and comparable to that obtained for the *Cish* promoter, a validated STAT 5 target[49]. In contrast, there was little detectable binding to either the *Rnu7* or α-*amylase* promoters.

We next determined if STAT 5B could attenuate E2A binding to $E_{\kappa i}$. *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells expressing CA-STAT 5B or control cells were cultured in the presence or absence of IL-7. Aliquots of each cell population were then assayed by quantitative ChIP for E47 binding to endogenous $E_{\kappa i}$. As demonstrated in Fig. 8e, withdrawal of IL-7 was associated with enhanced binding of E47 to $E_{\kappa i}$. This enhancement was attenuated by expression of CA-STAT 5B. These data are consistent with a model in which downstream of the IL-7R, STAT 5 inhibits *Ig*κ transcription by directly limiting the accessibility of $E_{\kappa i}$ to E2A.

DISCUSSION

It has been proposed that differentiation of pre-B cells into immature B cells occurs when they escape pro-proliferative IL-7R signals[22]. Our findings demonstrate that this is not sufficient. In addition, pre-BCR mediated Ras/MEK/ERK activation is required to orchestrate the principal molecular events of this developmental transition: cell cycle exit and *Ig*κ recombination (schematic model provided in Supplementary Fig. 7). ERK directs these outcomes by divergent mechanisms, utilizing lineage and developmentally restricted downstream effector molecules. The identification of a central, IL-7R modulated differentiation pathway provides a framework for understanding how signaling through the two principal receptors that dictate pre-B cell fate, the pre-BCR and IL-7R, coordinately regulate proliferation and differentiation. Furthermore, our findings provide an instructive example of how the molecular context of signal activation dictates cell fate.

A major function of the Ras/MEK/ERK pathway was to silence *Ccnd3* and terminate proliferation. This result was surprising as Ras has been demonstrated to induce proliferation in a variety of cell types[50]. However, the unique anti-proliferative effect of Ras in pre-B cells was dependent upon Aiolos, a lineage and developmentally restricted transcription factor[37], which coupled Ras activation to the silencing of *Ccnd3*. A central role for Aiolos in cell cycle termination is consistent with observations that *Aiolos*$^{-/-}$ mice have increased numbers of proliferating pre-B cells[39]. Furthermore, Aiolos is upregulated by expression of the pre-BCR[36–38]. In contrast to the necessity of Aiolos in regulating pre-B cell proliferation, the family member Ikaros[36] appeared to have no substantial role. Aiolos also suppresses SLC expression[37], indicating that it may silence a program of pre-B cell specific gene products.

While it is clear that Aiolos is required for Ras-mediated silencing of *Ccnd3*, it is likely that Ras is not the only inducer of Aiolos. Inhibiting Ras only partially attenuated *Aiolos* expression. The transcription factor IRF4 has also been shown to induce *Aiolos*[20] in pre-B cells. Although compromised, Aiolos induction in *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells is sufficient to allow cell cycle exit and *Ig*κ recombination.

In addition to silencing *Ccnd3*, Ras/MEK/ERK augmented *Ig*κ transcription by enhancing E2A expression and downregulating Id3. Ras/MEK/ERK only induced moderate changes in the level of each protein. However, this translated into a large increase in E2A binding activity. Among the Id proteins, Ras preferentially regulated Id3 and loss of Id3 was sufficient to dysregulate *Ig*κ expression. These findings reveal that there is some functional specificity among the Id proteins expressed during B lymphopoiesis. Our results are in contrast to previous observations that Ras induces *Id3* expression in double positive (DP)

thymocytes and attenuates E2A activity[51]. However, the Egr transcription factors which upregulate Id3 in thymocytes, were only transiently suppressed following MEK inhibition in *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells (data not shown). Therefore, it is not surprising that TCR dependent signals in DP thymocytes would regulate Id3 differently than signals through the pre-BCR.

Downstream of the Ras/MEK/ERK pathway, the specific molecular events of development were mediated by discrete and non-overlapping mechanisms. Aiolos repressed *Ccnd3* transcription but had no effect on cell cycle inhibitors or on the mediators of *Igκ* recombination. Conversely, E2A enhanced *Igκ* transcription but had no significant effect on the cell cycle machinery or on *Rag* expression. This latter finding is consistent with prior observations that E2A is required for initial *Rag* expression in pro-B cells but not for subsequent receptor-induced expression[52].

Proximal Ras/MEK/ERK activation coordinates a series of nuclear events required for development. This could potentially lead to a dangerous state as an aberration in one of the downstream effectors of ERK activation could uncouple cell cycle exit from *Igκ* recombination. However, RAG protein levels are tied to cell cycle progression as RAG and p27 are degraded through a common pathway[53]. It is likely that this, and potentially other mechanisms intrinsic to cell cycle progression[54], ensure genomic integrity.

Our observations may help resolve several apparent discrepancies in the literature concerning the role of Ras in lymphocyte development. Inhibiting the Ras/MEK/ERK pathway, either by expressing DN-Ras[16] or by lineage specific mutation of *Erk1* and *Erk2* induces a block at the pro-B cell stage[55]. In *Erk1*$^{-/-}$*Erk2*$^{-/-}$ mice, this is associated with a proliferative defect in the remaining CD19^{+}CD43^{+} cells and increased expression of SLC. In contrast, transgenic expression of CA-Ras throughout development induces extensive *Igκ* rearrangement[18]. We would argue that these two phenotypes arise from differential expression of Ras targets in pro and pre-B cells. In particular, the upregulation of Aiolos in pre-B cells is predicted to change the consequences of Ras activation. Other unexplored factors, such as differences in the amplitude or duration of Ras activation[56] in pro- and pre-B cells, may determine specific functional responses.

Tracing the signaling pathways downstream of Ras/MEK/ERK has revealed one mechanism for how the IL-7R suppresses pre-BCR-mediated differentiation. Ras activation greatly increases nuclear E2A binding activity. However, this is not sufficient to induce *Igκ* transcription as E2A cannot bind $E_{\kappa i}$ unless IL-7R signaling is attenuated. This suppressive function of the IL-7R is mediated by STAT 5 and is associated with the direct binding of STAT 5 to $E_{\kappa i}$. It is not clear how STAT 5 limits $E_{\kappa i}$ accessibility. However, STAT 5 has been demonstrated to be capable of both repressing[57] and activating[58] transcription. In addition to repressing *Igκ* transcription, STAT 5 coordinately enhanced *Ccnd3* transcription irrespective of pre-BCR signaling. The dominance of STAT 5 at the *Igκ* and *Ccnd3* loci provides a molecular explanation for why proliferation prevails over *Igκ* recombination when both the IL-7R and the pre-BCR are transmitting signals.

It is likely that the Ras/MEK/ERK signaling pathway also antagonizes the PI-3k/AKT signaling pathway. The latter favors proliferation over differentiation by suppressing *Rag* transcription[13,59] and by stabilizing cyclin D3 protein[30]. ERK may oppose the activities of Akt on *Rag* transcription as ERK can phosphorylate and activate the FoxO family members[60] which Akt target for degradation[13,59]. Our data demonstrate that ERK opposes the activities of PI-3k on cyclin D3 by silencing *Ccnd3* transcription. Additional undefined signaling pathways may influence cell cycle progression through the regulation of the inhibitors p21 and p27.

From our data, a picture emerges in which crucial molecular determinants of B lymphopoiesis are regulated by opposing signaling pathways that intersect at specific targets. By coordinately upregulating E2A and suppressing *Ccnd3* transcription, the Ras/MEK/ERK pathway plays a necessary and central role in the transition from proliferation to *Ig*κ recombination. This Ras-mediated differentiation program is modulated by STAT 5 which suppresses the activities of specific downstream ERK targets. It is through these, and other signaling networks[30,59], that the balance between IL-7R and pre-BCR signaling determines if a pre-B cell divides or undergoes *Ig*κ recombination.

METHODS

Mice

WT, *Irf4*$^{-/-}$*Irf8*$^{-/-}$, *Aiolos*$^{-/-}$ and *Id3*$^{-/-}$ (C57BL/6) and *Rag2*$^{-/-}$ (BALB/c) mice were housed in the animal facilities of the University of Chicago. Mice were used at 6–10 weeks of age and experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Chicago.

Isolation and culture of *Rag-2*$^{-/-}$ pro-B and *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells

CD19^{+} cells were isolated from bone marrow (6–10 week old mice) using a magnetic-activated cell sorter (MACS) separation column (Miltenyi Biotec). The resulting pro-B cells were cultured in complete Opti-MEM containing 7.5% fetal bovine serum and IL-7 (10ng/ml)[30]. Purified CD19^{+} cells from *Irf4*$^{-/-}$*Irf8*$^{-/-}$ mice were overlaid on OP9 stromal cells and cultured in complete medium with 7.5ng/ml of IL-7 (+IL-7) or 0.1ng/ml of IL-7 (−IL-7)[22]. Cells were typically >99% CD19^{+}, pre-BCR^{+}. For the inhibitor studies we used 10μM PD98059 (MEK inhibitor) and 5μM ERK inhibitor (both Calbiochem).

Retroviral gene transduction

cDNAs encoding murine CA-Ras (Ras61L), DN-Ras (RasN^{17}N), CA-STAT-5B (Dr. T. Gajewski, University of Chicago), DN-MEK (MEKK1-^{8}E-K^{97}M, Dr. Natalie G. Ahn, University of Colorado at Boulder), Aiolos, Igμ[32]or human ER-Id3 were sub-cloned into MIGR1. Myristylated Akt (CA-Akt) was used as described[61]. Control MIGR1 retrovirus or MIGR1 retroviruses containing a gene of interest were generated by calcium phosphate transfection of 293T cells and infection of the B cell progenitors was performed as described61. After 48 hours, GFP^{+} cells were isolated by FACS and cultured in complete medium.

Quantitative PCR analysis

Total cellular RNA was isolated with RNeasy kit (Qiagen) according to the manufacturer's protocol. RNA was reverse-transcribed with the Superscript III reverse transcriptase (Invitrogen). qPCR was performed in a total volume of 25μl containing 1μl cDNA template, 0.5 μM of each primer and SYBR Green PCRMaster Mix (Applied Biosystems). Gene expression was analyzed with the ABI PRISM 7300 Sequence Detector and ABI Prism Sequence Detection Software version 1.9.1 (Applied Biosystems). Sample results were normalized by division of the value for the unknown gene by that obtained for beta 2 microglobulin (*B2m*). Quadruplicate reactions were done with all cDNA samples. The primers used are presented in Supplementary Table 1.

Immunoblot

Total NP-40 cell lysates were prepared61 and 30μg of each lysate were resolved by 4–20% SDS-PAGE and then transferred to Immuno-Blot PVDF membranes (BioRad)[30]. Protein blots were probed with anti-Cyclin D3 (sc-182), anti-Cyclin D2 (sc-593), anti-Cyclin E

(sc-481) (all from Santa Cruz Biotechnology), anti-mouse Id3 (BD Pharmingen), anti-E47 (sc763, Santa Cruz Biotechnology), anti-total ERK (Zymed Laboratories), anti-phospho ERK (Phospho-p44/42 MAPK, Cell Signal), anti-STAT 5 (sc-835, Santa Cruz Biotechnology) and anti-Actin (Chemicon) as an internal loading control.

Flow cytometry

Single cell bone marrow suspensions were treated with Ack lysis buffer, resuspended in 3% FBS/PBS and stained with monoclonal antibodies to B220 (RA3–6B2), pre-BCR (SL156), CD43 (S7), CD25 (3C7), IgM (R6–60.2), CD11c (HL3), c-Kit(2B8), CD19 (1D3), NK1.1 (PK136), TCRβ (H57–597), Ter119 (TER-119), Mac-1 (M1/70), Gr-1 (RB6–8C5)(all from BD Biosciences Pharmingen). Anti-Igκ (H139-52.1) and its isotype Rat $IgG_{1\kappa}$ were from Southern Biotech. For intracellular staining with anti-phospho ERK antibodies (20A), cells were fixed with BD Cytofix buffer and permeabilized with BD Phosflow Perm Buffer III as recommended by the manufacturer (BD Biosciences Pharmingen). Monoclonal antibodies were directly coupled to fluorescein isothiocyanate, phycoerythrin, phycoerythrin-indotricarbocyanine, allophycocyanin or biotin. Surface marker expression on progenitor and mature B cells was acquired using a FACSCalibur (Becton Dickinson) and analyzed with FlowJo and CELLQuest software (Becton Dickinson). Cell sorting was done with a MoFlo cell sorter (Cytomation).

Cell Cycle Analysis

Cells were incubated in a solution containing propidium iodide and then analyzed on a FACScan (Becton Dickinson) as previously described[30]. Proportions of cells in G1, S, and G2/M phases of the cell cycle were analyzed using FlowJo and Cell Quest software (Becton Dickinson).

PCR analysis of *Ig*κ rearrangements

Semiquantitative PCR using genomic DNA was done as described[22]. For PCR analysis of *Ig*κ rearrangements indicated $Irf4^{-/-}Irf8^{-/-}$ cell populations were cultured in IL-7 as described for 48 hours. PCR reactions employed degenerate *V*κ and *Ig*κ intron primers[22] and 5-fold template dilutions. A region upstream of the *Ig*κ intron was amplified to control for amount of genomic DNA[22]. DNA from WT splenic IgM^+ B cells was used as positive control. PCR products were resolved by agarose gel electrophoresis, transferred to Hybond-N membranes (Amersham) and were quantitatively analyzed by Southern blotting using an *Ig*κ intron probe. Products were quantified using a Phosphoimager[22]. Primer information is provided in Supplementary Table 1.

Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared as described previously[22]. Prior to the addition of biotin-labeled double-strand DNA probe, 5μg nuclear extracts were incubated for 20 minutes on ice in 20 μl of reaction buffer containing 1X binding buffer, 1 μg of double-stranded poly(dI-dC), 2.5% glycerol, 0.05% NP-40 and 1 μg of bovine serum albumin. Samples were incubated with biotin-labeled probes (20 fmol/each) for 20 minutes at room temperature. In competition experiments, nuclear extracts were pre-incubated with 100-fold molar excess of unlabeled, double-stranded oligonucleotides for 20 minutes on ice. In super-shift experiments, the extracts were pre-incubated with antibodies anti-STAT 5 (sc-835 from Santa Cruz Biotechnology) or anti-E47 (sc763 from Santa Cruz Biotechnology) for 60 minutes on ice. Protein-DNA complexes were separated on 6% nondenaturing TBE gels (Invitrogen) and observed by the LightShift Chemiluminescent EMSA kit following the manufacturer's procedure (Pierce). Oligonucleotide probes used are provided in Supplementary Table 1.

Chromatin Immunoprecipitations (ChIP)

The assays were performed using the ChIP Assay Kit according to the manufacturer's instructions (Upstate) with some modifications[61]. STAT 5 was immunoprecipitated with the antibody sc-835x and E47 with sc763x (both from Santa Cruz Biotechnology). Quantitative Real time PCRs were then performed on the purified DNA using using the primers provided in Supplementary Table 1.

Statistical analysis

Data were analyzed by unpaired t test and analysis of variance followed by the test of least significant difference for comparisons within and between groups. All categories within each analyzed experimental panel were compared with significant p values (<0.05) provided. All p values <0.001 were rounded to facilitate comparisons between results.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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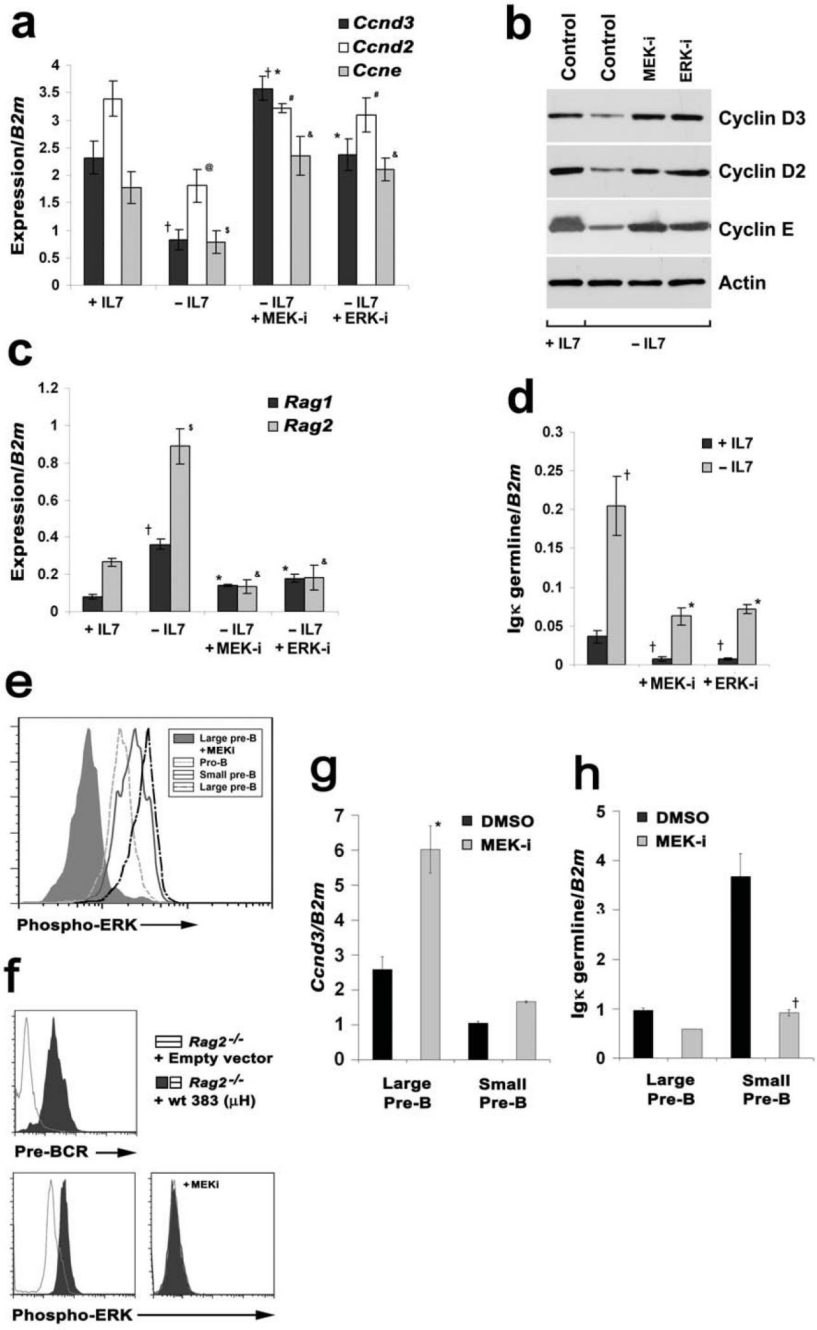

Figure 1. MEK/ERK regulate both cyclins and *Igκ* recombination machinery

(a) *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells were cultured in medium with (+ IL-7, 7.5ng/ml) or without (−IL-7, 0.1ng/ml) IL-7 for (a–b) 24 or (c–d) 48 hours. Cells were treated with either a MEK inhibitor PD98059 (MEK-i, 10μM) or an ERK inhibitor (ERK-i, 5μM) as indicated for the last six hours of culture. (**a**) Quantitative PCR (qPCR) for *Ccnd3*, *Ccnd2* and *Ccne* expression; †P<0.001 as compared with +IL-7 *Ccnd3* expression and *P<0.001 as compared with −IL-7 *Ccnd3* expression; $^{@}$P<0.001 as compared with +IL-7 *Ccnd2* expression and $^{\#}$P<0.001 as compared with −IL-7 *Ccnd2* expression; $^{\$}$P<0.001 as compared with +IL-7 *Ccne* expression and $^{\&}$P<0.001 as compared with −IL-7 *Ccne* expression. (**b**) Immunoblot for cyclin D3, cyclin D2 and cyclin E from *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B total cell lysates. Cells were

cultured according to the conditions described above. Actin immunoblot serves as an internal loading control. Analysis of three independent experiments indicated that expression of DN-Ras or DN-MEK increased cyclin D3 expression in −IL-7 cultures 4-fold as compared to untransfected controls (P<0.001, data not shown). (**c**) qPCR for *Rag1* and *Rag2* expression; †P<0.001 as compared with +IL-7 *Rag1* expression and *P<0.001 as compared with −IL-7 *Rag1* expression; $^{\$}$P<0.001 as compared with +IL-7 *Rag2* expression and $^{\&}$P<0.001 as compared with −IL-7 *Rag2* expression. (**d**) qPCR analysis for *Ig*κ germline transcription; †P<0.001 as compared with +IL-7 *Ig*κ germline transcription and *P<0.001 as compared with −IL-7 *Ig*κ germline transcription expression. For all quantitative real time PCR analysis, beta 2 microglobulin (*B2m*) was used as the endogenous reference gene and data represent mean ± SD from four independent experiments. (**e**) Pro-B cell ($B220^{+}CD43^{+}IgM^{-}$) or large and small pre-B cell ($B220^{+}CD43^{low/-}IgM^{-}$) populations were isolated by FACS, permeabilized, stained with anti-phospho-ERK antibodies and analyzed by flow cytometry. Indicated cells were treated with PD98059 (10μM) for 2 hours prior to analysis. (**f**) $Rag2^{-/-}$ pro-B cells were infected with either control MIG1 retrovirus or virus encoding Igμ. After 48 hours, indicated cell populations were surface stained with anti-pre-BCR antibodies (SL156, upper panel) or permeabilized and stained with anti-phospho-ERK antibodies (lower panels). In lower right panel, Igμ expressing cells were analyzed after incubation for 2 hours with PD98059. (**g** and **h**) Large and small WT pre-B cells were isolated by flow cytometry, cultured in either PD98059 or vehicle for six hours and analyzed by qPCR for the expression of (**g**) *Ccnd3* and (**h**) *Ig*κ germline transcription. *P<0.001 as compared with *Ccnd3* expression in large pre-B cells and †P<0.001 compared with κ germline transcription expression in small pre-B cells.

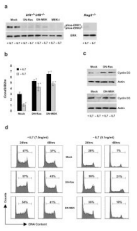

Figure 2. Downstream of pre-BCR, Ras-MEK activation required for cell cycle exit following IL-7 withdrawal

Irf4$^{-/-}$*Irf8*$^{-/-}$ pre-B cells expressing mock MIGR1 alone, DN-Ras or DN-MEK were cultured in medium with or without IL-7 for 24 or 48 hours as indicated or untransfected *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells were cultured with or without MEK–i for the last six hours of culture. (**a**) Immunoblot for phospho-ERK and total ERK using total cell lysates from *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells cultured according to the conditions described above and from *Rag2*$^{-/-}$ pro-B cells cultured in presence and absence of IL-7. Total-ERK immunoblot serves as an internal loading control. Data are representative of three independent experiments. (**b**) qPCR for *Ccnd3* expression; †P<0.001 as compared with +IL-7 *Ccnd3* expression and *P<0.001 as compared with −IL-7 *Ccnd3* expression. *B2m* was used as the endogenous reference gene. Data represent mean ± SD from four independent experiments. (**c**) Immunoblot for cyclin D3 from *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B total cell lysates. Cells were cultured according to the conditions described above. Actin immunoblot serves as an internal loading control. Data are representative of three independent experiments. (**d**) Cell cycle analysis of empty vector, DN-Ras or DN-MEK expressing *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells cultured as described. Data are representative of three independent experiments.

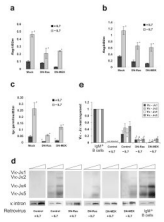

Figure 3. Ras-MEK activation is required for the induction of *Igκ* recombination following IL-7 withdrawal

Irf4$^{-/-}$*Irf8*$^{-/-}$ pre-B cells expressing mock MIGR1, DN-Ras or DN-MEK were cultured in medium with or without IL-7 for 48 hours. (**a–c**) qPCR for (a) *Rag1*, (b) *Rag2* and (c) *Igκ* germline expression. †P<0.001 as compared with mock infected +IL-7 (a) *Rag1*, (b) *Rag2* and (c) *Igκ* germline transcription and *P<0.001 as compared with −IL-7 (a) *Rag1*, (b) *Rag2* and (c) *Igκ* germline transcription. *B2m* was used as the endogenous reference gene. Data represent mean ± SD from four independent experiments. (**d**) Semi-quantitative PCR analysis of *Igκ* rearrangement in *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells cultured in medium as described for 48 hours. DNA from WT splenic IgM^{+} B cells was used as the positive control. PCR reactions employed degenerate Vκ primers and an *Igκ* intron primer (Supplementary Table 1) with 5 fold template dilutions. A region upstream of the *Igκ* intron was amplified to control for input genomic DNA. Amplified products were detected by hybridization with a k intron probe. Data are representative of three independent experiments. (**e**) Quantitative analysis of Vκ-Jκ rearrangement (shown in **d**). The relative intensities for Vκ-Jκ1, Vκ-Jκ2, Vκ-Jκ4, Vκ-J5κ, and input genomic DNA (control) were calculated by ImageJ software (NIH); values from rearrangements were divided by the control and then depicted within a bar diagram, setting the values of normalized Vκ-Jκ1, Vκ-Jκ2, Vκ-Jκ4 and Vκ-J5κ rearrangement in IgM^{+} pre-B cells as 1. *P<0.05 and †P<0.01 as compared with −IL-7 control Vκ-Jκ1 expression; $^{@}$P<0.01 and $^{\#}$P<0.001 as compared with −IL-7 control Vκ-Jκ2 expression; $^{\$}$P<0.05 and $^{\&}$P<0.01 as compared with −IL-7 control Vκ-Jκ4 expression and $^{\varphi}$P<0.01 as compared with −IL-7 control Vκ-Jκ5 expression. Data represent mean ± SD from three independent experiments.

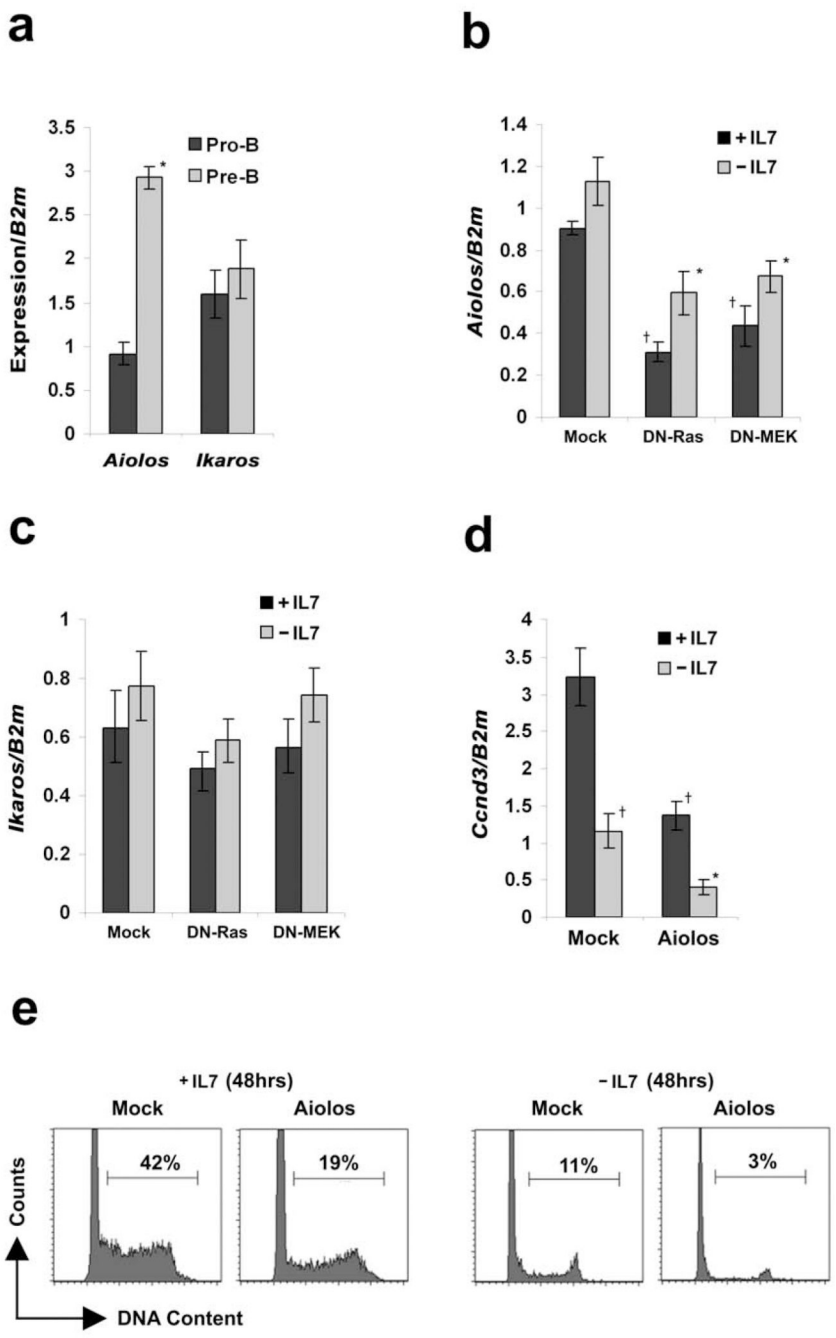

Figure 4. Aiolos is induced in pre-B cells and can suppress *Ccnd3* transcription

(**a**) WT pro-B cells ($CD19^{+}CD43^{high}IgM^{-}$) and pre-B cells ($CD19^{+}CD43^{low/-}IgM^{-}$) were isolated by flow sorting. The relative expression of *Aiolos* and *Ikaros* was analyzed by quantitative real time PCR. *P<0.001 as compared with *Aiolos* expression in pro-B cells. (**b–c**) qPCR analysis of (**b**) *Aiolos* and (**c**) *Ikaros* in $Irf4^{-/-}Irf8^{-/-}$ pre-B cells expressing vector alone, DN-Ras or DN-MEK cultured in medium with or without IL-7 for 24 hours. †P<0.001 as compared with vector alone +IL-7 *Aiolos* expression and *P<0.001 as compared with −IL-7 *Aiolos* expression. (**d**) qPCR analysis for *Ccnd3* expression in $Irf4^{-/-}Irf8^{-/-}$ pre-B cells expressing either MIGR1 or Aiolos cultured in medium with or without IL-7 for 24 hours. †P<0.001 as compared with vector infected +IL-7 *Ccnd3*

expression and *P<0.001 as compared with −IL-7 *Ccnd3* expression. *B2m* was used as the endogenous reference gene. Data represent mean ± SD from four independent experiments. (**e**) Cell cycle analysis of mock and Aiolos expressing *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells cultured in medium with or without IL-7 for 48 hours. Data are representative of three independent experiments.

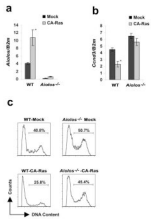

Figure 5. Downstream of Ras, Aiolos is required for suppressing *Ccnd3* and cell cycle exit following IL-7 withdrawal

Pre-B cells ($CD19^{+}CD43^{low}IgM^{-}$) were sorted from WT and $Aiolos^{-/-}$ mice and cultured for 24 hours in the presence of IL-7 (15ng/ml). Cells expressing either MIGR1 or CA-Ras were cultured in the presence of IL-7 (7.5ng/ml) for 48 hours. (**a–b**) The relative expression of (**a**) *Aiolos* and (**b**) *Ccnd3* in WT and $Aiolos^{-/-}$ pre-B cells expressing either mock or CA-Ras was analyzed by qPCR. (**a**) *P<0.001 as compared with *Aiolos* expression in mock infected wild-type pre-B cells and (**b**) *P<0.001 as compared with *Ccnd3* expression in mock infected wild-type pre-B cells. *B2m* was used as the endogenous reference gene. (**c**) Cell cycle analysis of mock and CA-Ras expressing WT and $Aiolos^{-/-}$ pre-B cells cultured with IL-7 for 48 hours.

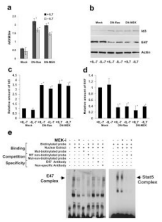

Figure 6. Downregulation of Id3 and upregulation of E2A by Ras-MEK signaling increases E2A binding activity

(**a**) qPCR analysis for expression of *Id3*. *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells expressing MIGR1, DN-Ras or DN-MEK were cultured in medium with or without IL-7 for 24 hours. †P<0.001 as compared with mock infected +IL-7 *Id3* and *P<0.001 as compared with −IL-7 *Id3*. *B2m* was used as the endogenous reference gene. Data represent mean ± SD from four independent experiments. (**b**) Western blot analysis of Id3 and the E2A protein E47 from indicated lysates of *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells expressing empty vector, DN-Ras or DN-MEK cultured in medium with or without IL-7 for 30 hours. Actin immunoblot serves as an internal loading control. Data are representative of three independent experiments. (**c–d**) The relative amount of (**c**) Id3 and (**d**) E47 normalized with actin in arbitrary units (a.u.) were calculated from western blots (n=3) by ImageJ software (NIH). *P<0.001 as compared with mock infected +IL-7 Id3 and E47 expression and †P<0.001 as compared with −IL-7 Id3 and E47 expression. (**e**) *In vitro* binding of E47 was assayed in EMSAs. Nuclear extracts were prepared from *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells cultured in medium with IL-7 for 30 hours and then treated with MEK inhibitor (MEK-i, 10μM of PD98059) for the last six hours of culture. An equal amount of nuclear proteins was used for each reaction. E2A binding was assayed with a probe corresponding to the E1 box of $E_{\kappa i}$. E47/DNA complexes were verified using an E47 antibody. STAT 5 protein/DNA complexes were assessed as a control.

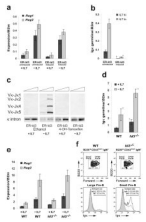

Figure 7. Increased *Igκ* transcription and rearrangement in *Id3*$^{-/-}$ mice

(**a–b**) qPCR analysis for (**a**) *Rag1* and *Rag2* and (**b**) *Igκ* germline expression in *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells expressing inducible *Id3* (ER-Id3-IRES-GFP) cultured in medium with or without IL-7 for 48 hours. Cells were either mock treated with ethanol (solvent of 4-OH-tamoxifen) or 4-OH-tamoxifen (1μM) to induce *Id3* expression. †P<0.001 as compared with mock infected +IL-7 *Igκ* germline transcription and *P<0.001 as compared with −IL-7 *Igκ* germline transcription. *B2m* was used as the endogenous reference gene. Data represent mean ± SD from four independent experiments. (**c**) Semi-quantitative PCR analysis of *Igκ* rearrangements in *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells expressing mock MIGR1 or ER-Id3 cultured as described above. DNA from WT splenic IgM^{+} B cells was used as the positive control. Data are representative of three independent experiments. (**d–e**) qPCR analysis for relative expression of (**d**) *Igκ* germline and (**e**) *Rag1* and *Rag2* transcription using sorted pre-B cells (CD19^{+}CD43$^{low/-}$IgM^{-}) from WT and *Id3*$^{-/-}$ bone marrow and cultured in medium with or without IL-7 for 48hrs. †P<0.001 as compared with wild-type +IL-7 *Igκ* germline transcription and *P<0.001 as compared with −IL-7 *Igκ* germline transcription. *B2m* was used as the endogenous reference gene. Data represent mean ± SD from three independent experiments. (**f**) Large and small pre-B cells (CD19^{+}CD43$^{low/-}$IgD^{-}) were sorted from WT and *Id3*$^{-/-}$ bone marrow and the surface expression of Igk was assayed by flow cytometry. Isotype control indicated. Data are representative of three independent experiments.

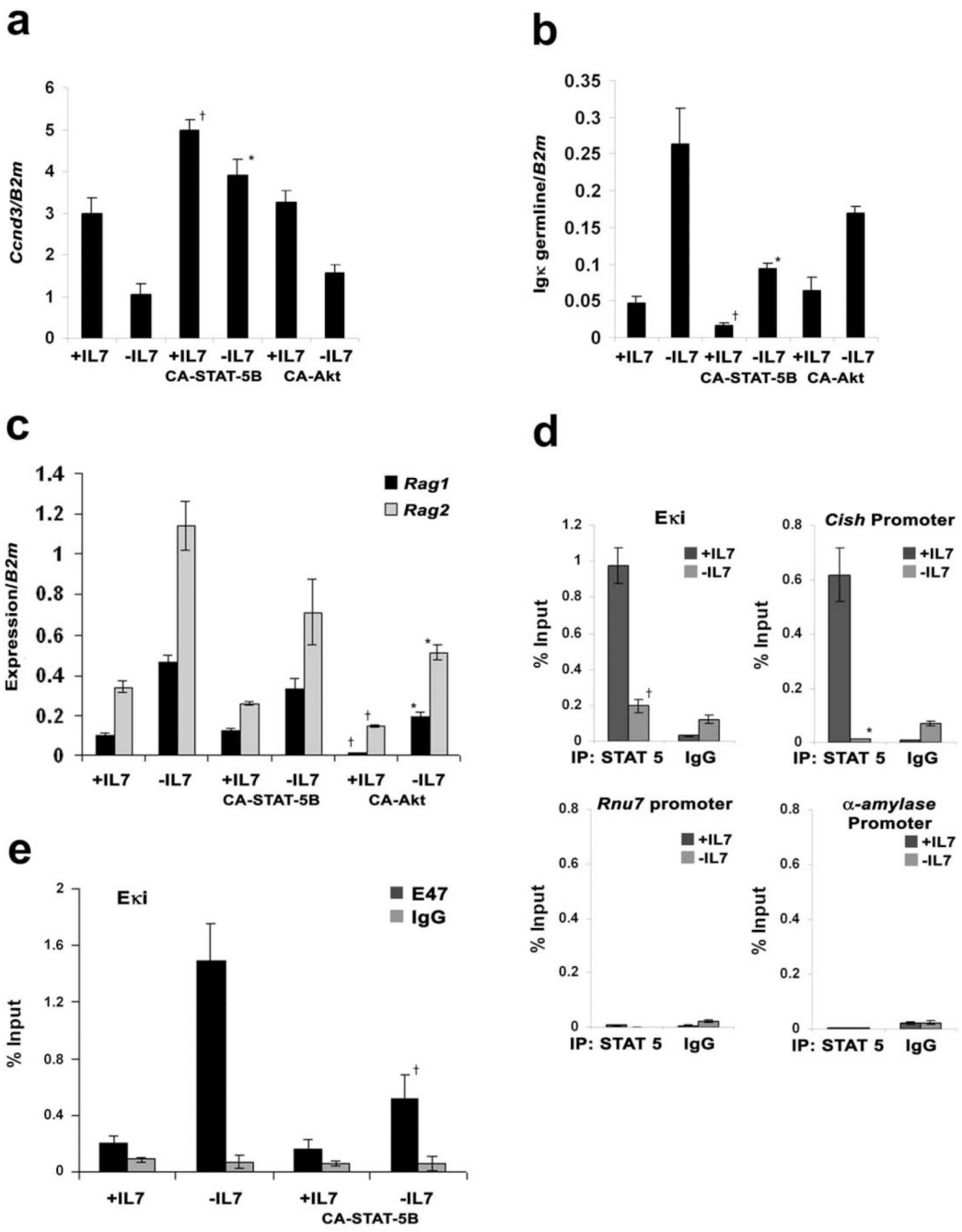

Figure 8. STAT 5 regulates *Igκ* transcription and binds directly to $E_{κi}$

Irf4$^{-/-}$*Irf8*$^{-/-}$ pre-B cells expressing MIGR1, CA-STAT 5B or CA-Akt were cultured in medium with or without IL-7 for (a) 24 and (b–d) 48 hours. (**a)** qPCR analysis for *Ccnd3* expression; †P<0.001 as compared with mock infected +IL-7 *Ccnd3* expression and *P<0.001 as compared with mock infected −IL-7 *Ccnd3* expression. *B2m* was used as the endogenous reference gene. Data represent mean ± SD from three independent experiments. (**b**) qPCR analysis for *Igκ* germline expression. †P<0.001 as compared with mock infected +IL-7 *Igκ* germline transcription and *P<0.001 as compared with mock infected −IL-7 *Igκ* germline transcription. *B2m* was used as the endogenous reference gene. Data represent mean ± SD from three independent experiments. (**c**) qPCR analysis shows *Rag1* and *Rag2*

expression. †P<0.001 as compared with mock infected +IL-7 *Rag1* (or *Rag2*) expression and *P<0.001 as compared with mock infected −IL-7 *Rag1* (or *Rag2*) expression. *B2m* was used as the endogenous reference gene. Data represent mean ± SD from three independent experiments. (**d**) *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells were cultured in medium with or without IL-7 for 48 hours and were subjected to ChIP assays with anti-STAT 5 antibodies. †P<0.001 as compared with binding of STAT 5 to E_{ki} with IL-7 and *P<0.001 as compared with binding of STAT 5 to *Cish* promoter. Data represent mean ± SD from three independent experiments. (**e**) *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells expressing CA-STAT 5B or vector alone were cultured in medium with or without IL-7 for 24 hours and were subjected to ChIP assays with anti-E47 antibodies. †P<0.001 as compared with binding of E47 to E_{ki} without IL-7.