Notch and the pre-TCR coordinate thymocyte proliferation by induction of the SCF subunits Fbxl1 and Fbxl12

Bin Zhao1, Kogulan Yoganathan2, LiQi Li1, Jan Y. Lee1, Juan Carlos Zúñiga-Pflücker2 and Paul E. Love3,*

Proliferation is tightly regulated during T cell development, and is limited to immature CD4−CD8+ thymocytes. The major proliferative event is initiated at the β-selection stage following successful rearrangement of TCRβ, and is triggered by and dependent on concurrent signaling by Notch and the pre-T cell receptor (TCR); however, it is unclear how these signals cooperate to promote cell proliferation. Here, we found that β-selection-associated proliferation required the combined activity of two Skp-cullin-F-box (SCF) ubiquitin ligase complexes that included as substrate recognition subunits the F-box proteins Fbxl1 or Fbxl12. Both SCF complexes targeted the cyclin-dependent kinase inhibitor Cdkn1b for polyubiquitination and proteasomal degradation. We found that Notch signals induced the transcription of Fbxl1, whereas pre-TCR signals induced the transcription of Fbxl12. Thus, concurrent Notch and pre-TCR signaling induced the expression of two genes, Fbxl1 and Fbxl12, whose products functioned identically but additively to promote degradation of Cdkn1b, cell cycle progression, and proliferation of β-selected thymocytes.

A major aspect of the thymocyte maturation process is the precise regulation of cell proliferation. Rather than being a shared property of all or most developing thymocytes, proliferation is strictly limited to two stages of early CD4−CD8− (double negative (DN)) thymocyte development. The initial proliferative phase, which occurs during the DN CD44+CD25− (DN1), DN CD44+CD25+ (DN2) and DN CD44−CD25− (DN3) stages before initiation of V(D)J recombination at the T cell receptor β (TCRβ) locus, is driven by thymus-expressed cytokines, specifically Kit ligand (stem cell factor) and interleukin-7 (IL-7), as well as signaling by Notch1−4. The second proliferative phase coincides with β selection7, so is initiated in DN3 cells that have productively rearranged TCRβ and express the pre-TCR. The proliferative burst that accompanies β selection takes place in DN CD44+CD25− (DN4) thymocytes, CD4−CD8− intermediate single-positive (ISP) thymocytes and early CD4+CD8+ (double positive (DP)) ‘blasts’ before rearrangement of TCRα, and is estimated to result in a 100−200-fold expansion5,6. The clonal expansion at this proliferative phase facilitates the diversification of the pre-selection TCR repertoire and is also required for the differentiation of DN thymocytes to the DP stage5.

Coordinated Notch-mediated and pre-TCR-mediated signaling is essential for β-selection-associated proliferation5,7, but precisely how the pre-TCR and Notch cooperate to regulate cell cycle entry and thymocyte proliferation has remained unclear5,8,9. Before pre-TCR expression, the majority of DN3 thymocytes are retained in either the quiescent G0 phase or the ‘primed’ G1 phase of the cell cycle10. Transition of cells from G0 to the actively cycling S/G2/M phases is controlled by cyclins complexed with a cyclin-dependent kinases (CDK)11. The activity of cyclin–CDK complexes, and consequently cell cycle progression, is inhibited by members of the Cip/Kip family of CDK inhibitors, which include Cdkn1a (p21Cip1), Cdkn1b (p27Kip1) and Cdkn1c (p57Kip2)11, with only Cdkn1b having a major role at the β-selection checkpoint12−16. Cdkn1b inhibits both cyclin A−CDK and cyclin E−CDK complexes17, and mice lacking Cdkn1b have an enlarged thymus18−20, whereas overexpression of Cdkn1b results in a block at the DN3 stage and a markedly reduced thymus size and cellularity21. Cdkn1b is highly expressed in quiescent ‘pre-β-selection’ DN3 thymocytes, but is downregulated at the initiation of β selection22.

Downregulation of Cdkn1b occurs primarily through its polyubiquitination by a SCF E3 ligase complex that includes the F-box substrate recognition protein Fbxl1, resulting in the proteasomal degradation of Cdkn1b23−25. Fbxl1−/− mice have a reduced thymus size, which is restored in Fbxl1−/−Cdkn1b−/− mice26. Notch signaling induces the expression of Fbxl1 in several cell lines, including T cell acute lymphoblastic leukemia cells, suggesting that Fbxl1 could be regulated by Notch in DN thymocytes27,28. Degradation of Cdkn1b also coincides with pre-TCR signaling29−31; however, a direct regulatory role for the pre-TCR in the destabilization of Cdkn1b has not been established.

In this study, we investigated the regulation of Cdkn1b stability and its effect on cell cycle progression and proliferation at the β-selection checkpoint. Our findings identified a key role for the F-box protein Fbxl1, and an equally critical role for the related F-box protein Fbxl12 in the destabilization of Cdkn1b. SCF complexes that contained Fbxl1 and SCF complexes that contained Fbxl12 cooperated in an additive fashion to target Cdkn1b for polyubiquitination and proteasomal degradation, and each was required for normal proliferation after β selection. Notably, Fbxl1 and Fbxl12 were induced transcriptionally at the β-selection checkpoint by Notch signals and pre-TCR signals, respectively. Together, these findings...
provide a regulatory mechanism for the β-selection proliferative burst that explains the requirement for and cooperativity of Notch and pre-TCR signaling for this response.

Results

Cdkn1b and Fbxl1 control β-selection proliferation. We generated Lck-Cre Cdkn1bfl/fl mice to induce deletion of Cdkn1b selectively in immature DN thymocytes. Similar to germline Cdkn1b−/− mice, thymus size and cellularity, as well as numbers of CD4+CD8− (CD4 single-positive (CD4 SP)) and CD4+CD8+ (CD8 single-positive (CD8 SP)) spleen T cells, were increased by approximately twofold in Lck-Cre Cdkn1bfl/fl mice compared with Lck-Cre Cdkn1b+/+ mice (Supplementary Fig. 1a–d and data not shown). The percentages of cycling DN4 thymocytes, ISP thymocytes and DP blasts were significantly increased (approximately 1.3-fold) in Lck-Cre Cdkn1bfl/fl mice compared with Lck-Cre Cdkn1b+/+ mice (Supplementary Fig. 2a). Most Lck-Cre Cdkn1bfl/fl DP thymocytes (non-blasting) were quiescent (G0, or G1 phase), similar to Lck-Cre Cdkn1b+/+ DP thymocytes (Supplementary Fig. 2a), indicating that they had successfully exited the cell cycle after the β-selection proliferative burst. Thus, deletion of Cdkn1b increased or extended post-β-selection proliferation, but did not induce proliferation in normally quiescent cell populations.

Germline deletion of the gene encoding Fbxl1 (Fbxl1)—the substrate recognition subunit of the SCF E3 ligase complex that targets Cdkn1b for polyubiquitination and proteasomal degradation is embryonic lethal23–25—in resulted in a partial DN3–DN4 developmental block and a twofold reduction in the total number of thymocytes and splenic CD4 SP and CD8 SP T cells compared with Fbxl1+ +/− mice (Supplementary Fig. 1a,c,d), confirming previous reports26. The percentages of cycling DN4 thymocytes, ISP thymocytes and DP blasts in Fbxl1−/− mice were reduced by twofold compared with Fbxl1+ +/− mice (Supplementary Fig. 2a). Apoptosis was not increased (Supplementary Fig. 2b), suggesting that the reduction in Fbxl1−/− thymocyte numbers was caused by reduced proliferation in response to β-selection signals. Fbxl1 interacted with cul1 (Cul1) and therefore functioned as a subunit of an SCF complex (hereafter, SCF-Fbxl1) (Supplementary Fig. 2c). Fbxl1 bound to and destabilized Cdkn1b (Supplementary Fig. 2d), and this activity required the F-box domain of Fbxl1 (Supplementary Fig. 2e)18–20. Consistent with these findings, expression of Cdkn1b was increased in DN thymocytes from Fbxl1−/− mice compared with Fbxl1+ +/− mice (Supplementary Fig. 2f). The partial DN3–DN4 block, reduction in thymocyte cellularity, and cell cycle defects were completely reversed in Lck-Cre Cdkn1bfl/fl Fbxl1−/− mice (Supplementary Figs. 1a,c,d and 2a), indicating that the developmental defects in Fbxl1−/− mice were caused by failure to downregulate Cdkn1b. Together, these findings demonstrate that β-selection-associated proliferation is regulated by SCF-Fbxl1-mediated degradation of Cdkn1b.

SCF-Fbxl12 regulates Cdkn1b and β-selection proliferation. Compared with the moderate developmental defects observed in Fbxl1−/− mice, transgenic overexpression of Cdkn1b results in an almost complete DN3–DN4 block and a tenfold reduction in thymocyte numbers27, suggesting that additional F-box protein(s) may regulate the turnover of Cdkn1b in immature thymocytes. Phylogenetic characterization, and sequence and motif comparison of mammalian F-box proteins, indicated that Fbxl12 is closely related to Fbxl1, suggesting that these proteins may target the same substrate28. Fbxl12 was highly and selectively expressed in thymocytes in both mice and humans (Supplementary Fig. 3a,b), and similar to Fbxl1, its expression was mostly limited to DN and DP thymocytes (Supplementary Fig. 3c). Co-transfection experiments in HEK-293T cells showed that, similar to Fbxl1, Fbxl12 bound to Cul1 (Fig. 1a), indicating that Fbxl12 functions as a subunit of an SCF complex (hereafter, SCF-Fbxl12). Fbxl12 also bound to Cdkn1b (Fig. 1b), and proteasome blockade with MG132 revealed that, similar to SCF-Fbxl1 complexes14–20, SCF-Fbxl12 complexes targeted Cdkn1b for polyubiquitination and proteasomal degradation (Fig. 1c,d), and that this activity required the F-box motif (Fig. 1d,e).

Germline deletion of Fbxl12 is embryonic lethal29. Therefore, we generated mice with a conditional (floxed) deletion allele of Fbxl12 (Fbxl12floxe) and crossed these to Lck-Cre transgenic mice to delete Fbxl12 selectively in DN thymocytes (Supplementary Fig. 3d–f). The phenotype of Lck-Cre Fbxl12floxe mice was similar to that of Fbxl1−/− mice; specifically, there was a substantial but incomplete block at the DN3–DN4 transition and an approximately twofold reduction in the number and percentage of cycling (S/G2/M phase) DN4, ISP and DP blasts compared with Lck-Cre Fbxl1+ +/− mice (Fig. 2a–d and Supplementary Fig. 4a). Numbers of DP thymocytes, CD4 SP and CD8 SP thymocytes and spleen T cells were also reduced approximately twofold in Lck-Cre Fbxl12floxe mice compared with Lck-Cre Fbxl12+ +/− mice (Fig. 2c and Supplementary Fig. 4b). Also similar to Fbxl1−/− mice, there was no increase in apoptosis of Lck-Cre Fbxl12+ +/− thymocytes compared with Lck-Cre Fbxl12+ +/− thymocytes (Supplementary Fig. 4c). Expression of the orphan nuclear retinoic acid-related orphan receptor γ t (ROsyt), which regulates the expression of Cdkn1b and cell cycle entry in DP thymocytes30, was unaffected in Lck-Cre Fbxl12+ +/− thymocytes (Supplementary Fig. 5a). Moreover, expression of a TCRβ transgene in Lck-Cre Fbxl12+ +/− thymocytes failed to reverse the partial DN3–DN4 block or restore normal thymocyte cellularity (Supplementary Fig. 5b,c), indicating that the developmental defect in Lck-Cre Fbxl12+ +/− thymocytes was not caused by a defect in TCRβ rearrangement. Expression of Cdkn1b was increased in both total (Supplementary Fig. 3f) and DN Lck-Cre Fbxl12+ +/− thymocytes (Fig. 2b) compared with Fbxl12+ +/− thymocytes. As observed with Fbxl1−/− mice, the developmental defects in Lck-Cre Fbxl12+ +/− thymocytes were reversed in Lck-Cre Cdkn1bfl/fl Fbxl12+ +/− thymocytes (Fig. 3a–c). These results indicate that, similar to Fbxl1, the primary function of Fbxl12 in thymocytes is to regulate the turnover of Cdkn1b.

SCF-Fbxl1 and SCF-Fbxl12 function additively. To determine whether deletion of both Fbxl1 and Fbxl12 exacerbated the reduction in β-selection-associated proliferation compared with the individual gene deletions, we generated Lck-Cre Fbxl12floxe Fbxl1−/− mice. These mice had normal frequencies (Fig. 4a) and numbers (Fig. 4b) of early DN1 and DN2 thymocytes, but had a profound block at the DN3–DN4 transition (Fig. 4a,c) and a much more severe reduction in the number and proliferation of DN4, ISP and DP blasts compared with either Fbxl1−/− or Lck-Cre Fbxl12+ +/− mice (Fig. 4c,d and Supplementary Fig. 6a), in addition to a significant further reduction in DP thymocytes, CD4 SP and CD8 SP thymocytes and spleen T cell counts (Fig. 4c and Supplementary Fig. 6b). Expression of Cdkn1b was increased further in Lck-Cre Fbxl12+ +/− Fbxl1−/− DN thymocytes compared with Fbxl1+ +/− or Lck-Cre Fbxl12+ +/− DN thymocytes (Fig. 4e), but did not result in an increase in thymocyte cell death (Supplementary Fig. 6c). To test whether the extent of β-selection-induced cell cycle progression and proliferation was sensitive to the amount of Fbxl1 and Fbxl12, we generated Lck-Cre Fbxl12+ +/− Fbxl1−/− mice in which Fbxl1 and Fbxl12 expression in DN thymocytes was reduced by approximately 50% compared with Fbxl12+ +/− Fbxl1+ +/− mice (Supplementary Fig. 7a,b). Lck-Cre Fbxl12+ +/− Fbxl1+ +/− DN thymocytes had increased expression of Cdkn1b compared with Fbxl12+ +/− Fbxl1−/− thymocytes (Supplementary Fig. 7b), and exhibited a phenotype that closely resembled that of Fbxl1−/− or Lck-Cre Fbxl12+ +/− mice (Supplementary Fig. 7a–d). These observations indicated that the proliferative response to β selection was sensitive to cellular amounts of Fbxl1, Fbxl12 and Cdkn1b.

SCF-Fbxl1 and SCF-Fbxl12 target the same site on Cdkn1b. Next, we examined the type and specificity of Cdkn1b polyubiquitination by SCF-Fbxl1 or SCF-Fbxl12 E3 ubiquitin ligase complexes. As expected, SCF-Fbxl1 and SCF-Fbxl12 each directed the lysine

articles

natureimmunology

1832

natureimmunology | VOL 20 | OCTOBER 2019 | 1381-1392 | www.nature.com/natureimmunology
Notch and pre-TCR regulate Fbxl1 and Fbxl12, respectively. Because proliferation of DN thymocytes at the β-selection checkpoint is dependent on coordinated signals transduced by Notch1 and the pre-TCR3,4,5,6,7, we next investigated whether these inductive signals regulated the expression of Fbxl1 and/or Fbxl12. To evaluate the impact of Notch signaling on the expression of Fbxl1 and Fbxl12, we cultured Rag2−/− DN3 thymocytes, which are pre-TCR−, on OP9 stromal cells transduced with the Notch ligand Delta-like 1 (OP9-DL1)8. Rag2−/− DN3 cells cultured on OP9-DL1 cells, but not on OP9 cells, upregulated Fbxl1 messenger RNA (mRNA) and proteins after 1 or 2 d of culture relative to day 0, and this was associated with a reduction in the expression of Cdkn1b (Fig. 6a,b). Induction of Fbxl1 mRNA in Rag2−/− thymocytes was observed 4 h after plating on OP9-DL1 cells (Supplementary Fig. 8a), indicating that this response did not require cell proliferation or transition to the DN4 stage. No increases in Fbxl1 mRNA or proteins were detected in Rag2−/− DN3 cells cultured on either OP9 or OP9-DL1 cells at any time point between 4 h and 2 d relative to day 0 (Fig. 6a,b and Supplementary Fig. 8a), indicating that Notch signaling induced the transcription of Fbxl1, but not Fbxl12. Injection of Rag2−/− mice, whose thymocytes have a complete block in development at the DN3 stage9,10, with monoclonal antibodies (mAbs) against CD3 mimics pre-TCR signaling by engagement of surface CD3 complexes that lack TCRβ and pre-TCRα, and induces a strong proliferative burst and transition of thymocytes to the DP stage11,12. Fbxl12 protein was modestly increased in (K)48 polyubiquitination of Cdkn1b (Fig. 5a,b)—a modification that targets proteins for proteasomal degradation13. Consistent with the previous characterization of K165, which is conserved in mouse and human Cdkn1b as the major, and possibly sole, site of Cdkn1b (OP9-DL1)13. Rag2−/− DN3 cells cultured on OP9-DL1 cells, but not on OP9 cells, upregulated Fbxl1 messenger RNA (mRNA) and proteins after 1 or 2 d of culture relative to day 0, and this was associated with a reduction in the expression of Cdkn1b (Fig. 6a,b). Induction of Fbxl1 mRNA in Rag2−/− thymocytes was observed 4 h after plating on OP9-DL1 cells (Supplementary Fig. 8a), indicating that this response did not require cell proliferation or transition to the DN4 stage. No increases in Fbxl1 mRNA or proteins were detected in Rag2−/− DN3 cells cultured on either OP9 or OP9-DL1 cells at any time point between 4 h and 2 d relative to day 0 (Fig. 6a,b and Supplementary Fig. 8a), indicating that Notch signaling induced the transcription of Fbxl1, but not Fbxl12. Injection of Rag2−/− mice, whose thymocytes have a complete block in development at the DN3 stage14,15, with monoclonal antibodies (mAbs) against CD3 mimics pre-TCR signaling by engagement of surface CD3 complexes that lack TCRβ and pre-TCRα, and induces a strong proliferative burst and transition of thymocytes to the DP stage16,17. Fbxl12 protein was modestly increased in
Rag2<sup>−/−</sup> total thymocytes on day 1 after intraperitoneal injection of CD3 mAbs, and was strongly increased on days 2 and 3 compared with total thymocytes from Rag2<sup>−/−</sup> mice that were not injected (Fig. 6c). Induction of Fbxl12 coincided with the downregulation of Cdkn1b (Fig. 6c) and CD25 (Fig. 6d) but preceded transition to the DP stage (Fig. 6c,d). Fbxl12 mRNA was induced in thymocytes 8 h after CD3 mAb injection, and continued to be induced at 24 h and on days 2 and 3 (Fig. 6e and Supplementary Fig. 8b). However, Fbxl1 mRNA and proteins were not upregulated in Rag2<sup>−/−</sup> thymocytes in response to intraperitoneal injection of CD3 mAbs (Fig. 6c,e and Supplementary Fig. 8b), indicating that pre-TCR signaling selectively induced the expression of Fbxl12. In addition, Rag2<sup>−/−</sup> DN3 thymocytes transduced with a TCRβ-IRE-GFP retrovirus (TCRβ tagged with internal ribosome entry site (IRES) and green fluorescent protein (GFP)), to induce the expression of the pre-TCR, and then cultured on OP9 cells expressing the Notch ligand DL4 (OP9-DL4 cells) upregulated Fbxl12 mRNA but not Fbxl1 mRNA compared with Rag2<sup>−/−</sup> DN3 thymocytes transduced.
with GFP retrovirus (Fig. 6f). These results showed that Notch and pre-TCR signals selectively induced the expression of Fbxl1 and Fbxl12, respectively.

**Fbxl1 and Fbxl12 can function interchangeably.** To test whether either Fbxl1 or Fbxl12 alone would be sufficient to promote normal β-selection-associated proliferation if expressed at sufficiently high...
levels, we transduced \textit{Fbxl1}−/− DN CD25−CD44−CD27hi (hereafter, DN3b) pre-TCR+ post-β-selected thymocytes with GFP retrovirus (GFP) or \textit{Fbxl12}-IRES-GFP (Fbx12-GFP) retrovirus, to increase the amount of Fbxl12 protein in DN3b thymocytes lacking Fbxl1, as well as \textit{Lck-Cre Fbxl12}−/− DN3b thymocytes with GFP retrovirus or \textit{Fbxl1-IRES-GFP (Fbx12-GFP)} retrovirus, to increase the amount of Fbx1
protein in DN3b thymocytes lacking Fbxl12, and then cultured the transduced cells for 3d on OP9-DL1 stromal cells. GFP-transduced Fbxl1 or Fbxl12 complexes in HEK-293T cells transfected with plasmids encoding HA-Ub, Flag-Cdkn1b or Flag-Cdkn1b(K165R) and Myc-Fbxl1 or Myc-Fbxl12 for 48 h, suggesting that forced expression of Fbxl1 or Fbxl12 could promote cell cycle progression and proliferation in the absence of Notch signals and pre-TCR signals, respectively, but were unable to substitute for Notch or the pre-TCR to promote the generation of DP thymocytes.

\[ \text{γδTCR}^- \text{thymocyte proliferation is controlled by Fbxl12. In contrast with αβ-lineage DN thymocytes, which require both Notch and pre-TCR signals for maturation to the DP stage and for normal β-selection-associated proliferation, immature CD24^hi γδ-lineage thymocytes are less responsive to (and dependent on) Notch signaling for maturation and proliferation}^{32,39}. \]

To examine the role of Fbxl1 and Fbxl12 in the proliferation of γδ-lineage thymocytes, we first evaluated the effect of Cdkn1b deletion on cell cycle progression and the proliferation of immature CD24^hi γδTCR^-thymocytes. The percentages of cycling CD24^hi γδTCR^-thymocytes, as well as the total numbers of γδTCR^-thymocytes, were decreased by approximately 1.5–2-fold in both Fbxl1^-/- and Lck-Cre Fbxl12^fl/fl mice compared with Lck-Cre Cdkn1b^+/+ mice. In contrast, the percentages of cycling CD24^hi γδTCR^-thymocytes, as well as the total numbers of γδTCR^-thymocytes, were decreased by approximately 1.5–2-fold in both Fbxl1^-/- and Lck-Cre Fbxl12^fl/fl mice compared with Lck-Cre Cdkn1b^+/+ mice. Notably, the reductions in percentage cycling and total numbers of γδTCR^-thymocytes were more severe in Lck-Cre Fbxl12^fl/fl mice compared with Fbxl1^-/- mice (Fig. 8c,d), suggesting that proliferation of immature γδ-lineage committed thymocytes was less dependent on Fbxl1 (Notch signaling) than on Fbxl12 (TCR signaling). Consistent with this observation, Fbxl1 mRNA was only slightly induced in γδTCR^- thymocytes cultured on OP9-DL1 cells compared with γδTCR^- thymocytes cultured on OP9 cells (Fig. 8e). We also detected little or no induction of Fbxl1 mRNA in γδTCR^- thymocytes cultured on either OP9 or OP9-DL.
Fbxl12 expression is regulated quantitatively by TCR signal strength dependent on Notch signaling-mediated induction of Fbxl1. Results show that the proliferation of γδ TCR induce the expression of the autonomously signaling pre-TCR. Both Fbxl12 mRNA expression (left) and Fbxl12 expression (right) in Rag2−/− DN3 thymocytes transduced with retrovirus encoding GFP or TCR β-transduced and KN6 TCR-transduced γδ TCR- and pre-TCR−/− thymocytes (Fig. 8f), suggesting that Fbxl12 was sufficient to elicit a normal proliferative response in DN3 thymocytes in the absence of the other F-box protein. These results support a model where Notch-induced Fbxl12 and less dependent on Notch signaling-mediated induction of Fbxl1.

Discussion

In this study, we demonstrated that two distinct SCF E3 complexes containing different F-box subunits (Fbx11 or Fbxl12) were required for the normal proliferative response of αβ-lineage (DN3 pre-TCR+) thymocytes at the β-selection checkpoint. Transcription of Fbxl11 was regulated by Notch signaling, whereas transcription of Fbxl12 was regulated by pre-TCR signaling, and SCF-Fbxl1 and SCF-Fbxl12 complexes each directed the polyubiquitination of the cyclin-dependent kinase inhibitor Cdkn1b. The combined activity of the SCF-Fbxl1 and SCF-Fbxl12 complexes was required to elicit the appropriate (normal) proliferative response to β-selection, as the absence of either Fbxl1 or Fbxl12 significantly and similarly attenuated (and the absence of both profoundly blocked) proliferation in DN4, ISP and DP-blast thymocyte populations. The requirement for both SCF-Fbxl1 and SCF-Fbxl12 complexes was quantitative, because both were necessary for β-selection-associated proliferation and targeted the same amino acid residue in Cdkn1b (K165) for polyubiquitination. Moreover, if highly expressed, either Fbxl1 or Fbxl12 was able to direct the complete degradation of cellular Cdkn1b in cell lines, and enhanced expression of either Fbxl1 or Fbxl12 was sufficient to elicit a normal β-selection-associated proliferative response in DN3 thymocytes in the absence of the other F-box protein. These results support a model where Notch-induced Fbxl1 and pre-TCR-induced Fbxl12 function identically, but also additively, to degrade Cdkn1b to an extent necessary for optimal β-selection-associated proliferation.
Fig. 7 | Fbxl1 and Fbxl12 function interchangeably to promote proliferation, but are not sufficient for β selection. a, Flow cytometry analysis showing the generation of DP thymocytes by DN3b thymocytes from mice of the indicated genotype transduced with retrovirus encoding GFP, Fbxl1-IRES-GFP or Fbxl12-IRES-GFP, and plated on OP9-DL1 cells for 3 d. One representative of four experiments is shown. b, Quantification of the results from the experiments shown in a. Left to right: numbers of total thymocytes; percentages of DP thymocytes; percentages of cycling S/G2/M DN cells; and percentages of cycling S/G2/M DP cells. c, Flow cytometry analysis showing the generation of DP thymocytes by DN3b thymocytes from B6 (wild-type) mice transduced with retrovirus encoding GFP (control) or Fbxl1-IRES-GFP, and plated on OP9 or OP9-DL1 cells for 3 d. d, Numbers of total thymocytes (left) and percentages of DP-stage cells (right) from the experiment in c. e, Flow cytometry analysis showing the generation of DP thymocytes from DN3b thymocytes from Rag2<sup>−/−</sup> (B6) mice transduced with retrovirus encoding GFP or Fbxl12-IRES-GFP, and plated on OP9-DL1 cells for 3 d. f, Numbers of total thymocytes (left) and percentages of DP-stage cells (right) from the experiment in e. For all graphs, central horizontal lines indicate means, while vertical lines indicate s.d. P values were determined by unpaired two-tailed Student’s t-test. *P < 0.05; **P < 0.01; ***P < 0.005; ****P < 0.0001. The data in b, d and f are the combined results of three experiments. The data in a, c and e are representative of three independent experiments.
Fig. 8 | Proliferation of immature γδ TCR+ thymocytes is mediated primarily by TCR-induced regulation of Fbxl12.  

a. Flow cytometry analysis showing total γδ TCR+ thymocytes (left) and the percentages of immature CD24hi γδ TCR+ thymocytes (centre) from the indicated mice. The right panels show the percentages of cycling S/G2/M CD24hi γδ TCR+ thymocytes.  

b. Quantitation of the percentages of cycling S/G2/M γδ TCR+ thymocytes (left) and numbers of total γδ TCR+ thymocytes (right).  

c. Flow cytometry analysis showing total γδ TCR+ thymocytes from mice of the indicated genotype (left) and percentages of immature CD24hi γδ TCR+ thymocytes (centre) from the indicated mice. The right panels show the percentages of cycling S/G2/M γδ TCR+ thymocytes.  

d. Quantitation of the percentages of cycling S/G2/M γδ TCR+ thymocytes (left) and numbers of total γδ TCR+ thymocytes (right).  

e. Real-time PCR analysis showing quantitation of Fbxl1 (left) and Fbxl12 (right) mRNA in total γδ TCR+ thymocytes from B6 (wild-type) mice plated on OP9 or OP9-DL1 cells for the indicated days. mRNA expression is relative to day 0.  

f. Real-time PCR analysis showing quantitation of Fbxl12 mRNA in Rag2−/−DN3 thymocytes transduced with retrovirus encoding GFP, TCR β-GFP or (KN6)TCR γδ-GFP, then plated on OP9-DL4 cells for the indicated time points. mRNA expression is relative to the mock-infected sample at 36 h. For all graphs, central horizontal lines indicate means, while vertical lines indicate s.d. P values were determined by unpaired two-tailed Student’s t-test. *P < 0.05; **P < 0.01; ***P < 0.005; ****P < 0.0001. The data in a and c are representative of three independent experiments. The data in e and f are the combined results of three experiments.
Cell cycling and proliferation in DN4, ISP and DP blasts was significantly attenuated in Fbxl11−/− Fbxl12−/− mice, in which the expression of Fbxl1 and Fbxl12 is reduced by approximately 50%, and the expression of Cdkn1b is increased by approximately two-fold, compared with Fbxl11−/− Fbxl12−/− mice, indicating that the proliferative response was highly sensitive to cellular amounts of Fbxl1, Fbxl12 and Cdkn1b. These results are concordant with reports that a twofold reduction in Cdkn1b is sufficient to induce cell cycle progression in peripheral CD4 SP T cells34. Both SCF-Fbxl1 and SCF-Fbxl12 complexes have been reported to target several other proteins in addition to Cdkn1b7,43-46. Germline deficiency of Fbxl12 is embryonic or perinatal lethal, and this has been attributed to trophoblast defects secondary to a lack of SCF-Fbxl12 complex-mediated degradation of placent aldehyde dehydrogenase 3 (ref. 27). However, it is notable that T cell development was effectively restored in both Fbxl11−/− and Lck-Cre Fbxl12−/− mice by the deletion of Cdkn1b, suggesting that Cdkn1b is the primary target of both SCF-Fbxl1 and SCF-Fbxl12 complexes relevant to β-selection-associated proliferation.

Although γδ-lineage (γ6TCR+) thymocytes were previously thought to be relatively quiescent8, recent data based on cell cycle analysis have identified similar rates of proliferation in immature γδ-lineage (pre-TCR+) and ligand-engaged γ6TCR+ thymocytes, suggesting that the reduced number of γδ-lineage thymocytes relative to γδ-lineage thymocytes is rather explained by the lower frequency of in-frame γδ rearrangement compared with β rearrangement, and the requirement for ligand-mediated signaling by the γ6TCR but not the pre-TCR9. Whereas Notch signals are required for early (DN1–DN3) thymocyte development10 and for the DN–DP developmental transition, regardless of the TCR complex expressed, most γδ-lineage committed thymocytes are relatively unresponsive to Notch ligands and can complete their maturation in the absence of Notch signaling11-13. Our observations that the proliferation of γ6TCR+ thymocytes was less impacted by the deletion of Fbxl1 than by the deletion of Fbxl12, and that, in contrast with pre-TCR+ thymocytes, γ6TCR+ thymocytes did not upregulate Fbxl1 on OP9-DL cells, are consistent with the idea that γ6TCR+ thymocytes are relatively unresponsive to Notch ligands. However, we found that the induction of Fbxl12 in response to ligand-mediated γ6TCR signaling was superior to that elicited by pre-TCR signaling, explaining how ligand-engaged γδ-lineage thymocytes can initiate a relatively robust proliferative response in the absence of Notch signaling.

In the absence of Cdkn1b, DP thymocytes successfully exit the cell cycle and become quiescent, indicating that distinct molecular mechanisms are involved in cell cycle regulation and proliferation in DN and DP thymocytes. In the absence of the orphan nuclear receptor RORyt, β selection appears to be unaffected, but DP thymocytes fail to exit the cell cycle and undergo apoptosis40. Deletion of the SCF F-box subunit Fbxw7 also does not affect DN thymocyte proliferation or β selection, but instead results in increased DP thymocyte proliferation and failure to exit the cell cycle as a result of elevated c-Myc41. Thus, whereas destabilization of Cdkn1b is the critical mediator of β-selection-induced proliferation in DN and ISP thymocytes and DP blasts, other regulatory proteins that include RORyt and Fbxw7 function as the key factors that enforce cell cycle exit and quiescence in DP thymocytes.

In summary, we identified a crucial role for destabilization of the cyclin-dependent kinase inhibitor Cdkn1b for the proliferative burst that occurs in response to β selection. Our results also show that cellular levels of Cdkn1b are controlled by the combined activity of two SCF ubiquitin ligase complexes (SCF-Fbxl1 and SCF-Fbxl12) that are independently regulated by Notch and pre-TCR signals, respectively, explaining the requirement for coordinated Notch and pre-TCR signaling for optimal thymocyte proliferation and differentiation at the β-selection checkpoint.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41590-019-0469-z.

Received: 31 October 2018; Accepted: 16 July 2019;
Published online: 26 August 2019

References
1. Rodewald, H. R., Ogawa, M., Haller, C., Waskow, C. & Di Santo, J. P. Pro-thymocyte expansion by c-kit and the common cytokine receptor y chain is essential for repertoire formation. Immunity 6, 265–272 (1997).
2. Shortman, K., Eggert, M., Spangrude, G. J. & Scolay, R. The generation and fate of thymocytes. Semin. Immunol. 2, 3–12 (1990).
3. Song, G. W., Knowles, G. C., Mak, T. W., Ferrando, A. A. & Zuniga-Pflucker, J. C. HES1 opposes a PTEN-dependent check on survival, differentiation, and proliferation of TCRβ-selected mouse thymocytes. Blood 120, 1439–1448 (2012).
4. Petit, C., Lucas, B. & Vasseur, F. Cell expansion and growth arrest phases during the transition from precursor (CD48−) to immature (CD48+) thymocytes in normal and genetically modified mice. J. Immunol. 154, 5103–5113 (1995).
5. Kreslavsky, T. et al. β-Selection-induced proliferation is required for output T cell differentiation. Immunity 37, 840–853 (2012).
6. Ciofani, M. et al. Obligatory role for cooperative signaling by pre-TCR and Notch during thymocyte differentiation. J. Immunol. 172, 5230–5239 (2004).
7. Hoffman, E. S. et al. Productive T-cell receptor β-chain gene rearrangement: coincident regulation of cell cycle and clonality during development in vivo. Genes Dev. 10, 948–962 (1996).
8. Maillard, I. et al. The requirement for Notch signaling at the β-selection checkpoint in vivo is absolute and independent of the pre-T cell receptor. J. Exp. Med. 203, 2239–2245 (2006).
9. Yasuhro-Ohmato, Y., Ohtani, T. & Pear, W. S. Notch regulation of early thymocyte development. Semin. Immunol. 22, 261–269 (2010).
10. Aifantis, I., Mandal, M., Sawai, K., Ferrando, A. & Vilimas, T. Regulation of T-cell progenitor survival and cell-cycle entry by the pre-T-cell receptor. ImmunoL Rev. 209, 159–169 (2006).
11. Rowell, E. A. & Wells, A. D. The role of cyclin-dependent kinases in T-cell development, proliferation, and function. Crit. Rev. Immunol. 26, 189–212 (2006).
12. Deng, C., Zhang, P., Harper, J. W., Elledge, S. J. & Leder, P. Mice lacking p21(Cip1)/p27Kip1 undergo normal development, but are defective in G1 checkpoint control. Cell 82, 675–684 (1995).
13. Matsumoto, A., Takeishi, S. & Nakayama, K. I. p57 regulates T-cell development and prevents lymphomagenesis by balancing p53 activity and pre-TCR signaling. Blood 123, 3429–3439 (2014).
14. Nakayama, K. et al. Mice lacking p27Kip1 display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. Cell 85, 707–720 (1996).
15. Kiyokawa, H. et al. Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27Kip1. Cell 85, 721–732 (1996).
16. Fero, M. L. et al. A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27Kip1−/− deficient mice. Cell 82, 733–744 (1996).
17. Tsukiyama, T. et al. Down-regulation of p27(Kip1) expression is required for development and function of T cells. J. Immunol. 166, 304–312 (2001).
18. Carrano, A. C., Eytan, E., Hershko, A. & Pagano, M. SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. Nat. Cell Biol. 1, 193–199 (1999).
19. Nakayama, K. et al. Skp2-mediated degradation of p27 regulates progression into mitosis. Dev. Cell 6, 661–672 (2004).
20. Kossatz, U. et al. Skp2-dependent degradation of p27 is essential for cell cycle progression. Genes Dev. 18, 2602–2607 (2004).
21. Sarmento, L. M. et al. Notch1 modulates timing of G1-S progression by inducing SKP2 transcription and p27Kip1 degradation. J. Exp. Med. 202, 157–168 (2005).
22. Dohda, T. et al. Notch signaling induces SKP2 expression and promotes reduction of p27Kip1 in T-cell acute lymphoblastic leukemia cell lines. Exp. Cell Res. 313, 3141–3152 (2007).
23. Del Dubbo, C. B. et al. Notch signaling activates stem cell properties of Müller glia through transcriptional regulation and Skp2-mediated degradation of p27Kip1. PLoS ONE 11, e0152025 (2016).
24. Hristova, N. R., Tagscherer, K. E., Fassl, A., Kopitz, J. & Roth, W. Notch1-dependent regulation of p27 determines cell fate in colorectal cancer. Int. J. Oncol. 43, 1967–1975 (2013).
25. Tsvetkov, L. M., Yeh, K. H., Lee, S. J., Sun, H. & Zhang, H. p27Kip1 ubiquitination and degradation is regulated by the SCFγδ complex through phosphorylated Thr187 in p27. Curr. Biol. 9, 661–664 (1999).
26. Jin, J. et al. Systematic analysis and nomenclature of mammalian F-box proteins. *Genes Dev.*, **18**, 2573–2580 (2004).
27. Nishiyama, M., Nita, A., Yumimoto, K. & Nakayama, K. I. FBXL12-mediated degradation of ALDH13 is essential for trophoblast differentiation during placental development. *Stem Cells*, **33**, 3327–3340 (2015).
28. Sun, Z. et al. Requirement for RORγt-mediated signals rescue the development of CD3ε-mediated signals to restore the development of T-cell receptor beta-chain-deficient mutant mice by transmembrane signaling through CD3 epsilon. *Immunity*, **20**, 749–756 (2002).
29. Hayes, S. M. & Love, P. E. Strength of signal: a fundamental mechanism for differential synergy of Notch and T cell receptor signaling determines ζβ versus γδ lineage fate. *J. Exp. Med.*, **203**, 1579–1590 (2006).
30. Schmitt, T. M. & Zuniga-Pflucker, J. C. Induction of T cell development from hematopoietic progenitor cells by Delta-like-1 in vitro. *Immunity*, **17**, 749–756 (2002).
31. Shinkai, Y. et al. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell*, **68**, 855–867 (1992).
32. Garbe, A. I., Krueger, A., Gounari, F., Zuniga-Pflucker, J. C. & von Boehmer, H. Differential synergy of Notch and T cell receptor signaling determines ζβ versus γδ lineage fate. *J. Exp. Med.*, **203**, 1579–1590 (2006).
33. Levelt, C. N., Mombaerts, P., Iglesias, A., Tonegawa, S. & Eichmann, K. Restoration of early thymocyte differentiation in T-cell receptor beta-chain-deficient mutant mice by transmembrane signaling through CD3 epsilon. *Proc. Natl Acad. Sci. USA*, **90**, 11401–11405 (1993).
34. Shinkai, Y. & Alt, F. W. CD3ε-mediated signals rescue the development of CD4+CD8+ thymocytes in RAG-2−/− mice in the absence of TCR β chain expression. *Immunity*, **6**, 995–1001 (1994).
35. Wiest, D. L., Kearse, K. P., Shores, E. W. & Singer, A. Developmentally regulated expression of CD3 components independent of donutopic T cell antigen receptor complexes on immature thymocytes. *J. Exp. Med.*, **180**, 1375–1382 (1994).
36. Taghon, T., Yui, M. A., Pant, R., Diamand, R. A. & Rothenberg, E. V. Developmental and molecular characterization of emerging β- and γδ-selected pre-T cells in the adult mouse thymus. *Immunity*, **24**, 53–64 (2006).
37. Ciofani, M., Knowles, G. C., Wiest, D. L., von Boehmer, H. & Zuniga-Pflucker, J. C. Stage-specific and differential Notch dependency at the ζβ and γδ T lineage bifurcation. *Immunity*, **25**, 105–116 (2006).
38. Haks, M. C. et al. Attenuation of p53 signaling efficiently diverts thymocytes to the ζβ lineage. *Immunity*, **18**, 595–606 (2005).
39. Hayes, S. M. & Love, P. E. Strength of signal: a fundamental mechanism for cell fate specification. *Immunol. Rev.*, **209**, 170–175 (2006).
40. Rowell, E. A., Walsh, M. C. & Wells, A. D. Opposing roles for the cyclin-dependent kinase inhibitor p27kip1 in the control of CD4+ T cell proliferation and effector function. *J. Immunol.*, **174**, 3359–3368 (2005).
41. Jiang, H. et al. Ubiquitylation of RAG-2 by Skp2-SCF links destruction of the V(D)J recombinase to the cell cycle. *Mol. Cell*, **18**, 699–709 (2005).
42. Tedesco, D., Lukas, J. & Reed, S. I. The p73-related protein p130 is regulated by phosphorylation-dependent proteolysis via the protein-ubiquitin ligase SCFγδ. *Genes Dev.*, **16**, 2946–2957 (2002).
43. Kim, S. Y., Herbst, A., Tworowski, K. A., Salghetti, S. E. & Tansey, W. P. Skp2 regulates Myc protein stability and activity. *Mol. Cell*, **11**, 1177–1188 (2003).
44. Passoni, L. et al. Intrathymic δ selection events in γδ cell development. *Immunity*, **7**, 83–95 (1997).
45. Prinz, I. et al. Visualization of the earliest steps of γδ T cell development in the adult thymus. *Nat. Immunol.*, **7**, 995–1003 (2006).
46. Wilson, A., MacDonald, H. R. & Radtke, F. Notch 1-deficient common lymphoid precursors adopt a B cell fate in the thymus. *J. Exp. Med.*, **194**, 1003–1012 (2001).
47. Onoyama, I. et al. Conditional inactivation of Fbxw7 impairs cell-cycle exit during T cell differentiation and results in lymphomatogenesis. *J. Exp. Med.*, **204**, 2875–2888 (2007).
Methods

Mice. Fbxl12 conditional knockout mice were generated with a targeting vector purchased from the Knockout Mouse Project repository (http://www.komp.org). The vector was linearized and transfected into B6 embryonic stem cells. Transfected embryonic stem cells were cultured with media containing neomycin, and resistant clones were screened for homologous recombination by PCR. Blastocyst injections resulted in several chimeric mice, three of which gave germline transmission. Germline Fbxl12+/- mice were crossed with ROSA26:FP+P mice (stock number 003946; Jackson Laboratory) to delete the Neo gene. Offspring were then crossed to generate Fbxl12+/- mice. Fbxl12+/- mice were provided by L. Zhu (Albert Einstein College of Medicine). Cdkn1a-/- mice were purchased from the Jackson Laboratory (stock number 027328). Lck-Cre transgenic mice, and TCR-transgenic mice, Rag2-/- mice and CD45.1 C57BL/6 mice were obtained from Taconic Biosciences. Animal experiments were approved by the Animal Care and Use Committee of the National Institute of Child Health and Human Development.

Cell lines. HEK-293T cells (American Type Culture Collection) and the Platinum-E Retroviral Packaging Cell Line (Cell Biolabs) were cultured in DMEM medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 μU/ml pencillin and 50 μg/ml streptomycin. OP9 cells expressing the Notch ligand Delta-like 1 (OP9-DL1) or Delta-like 4 (OP9-DL4), which were generated as previously described, were cultured in MEM medium supplemented with 5% fetal bovine serum (Gibco) and antibiotics (pencillin (100 μg/ml) + streptomycin (100 μg/ml); Invitrogen; OP9 media). The GFP-TCRγδ, -TCRβ, -Fbxl12, -Fbxl12 retrovirus-producing GP + E cell lines were generated using pMIG-RES-GFP as previously described.

Plasmids and constructs, and retroviral transduction. Fbxl12 was amplified from B6 thymocyte complementary DNA (cDNA) and cloned into pRES-hrGFP-2a (Agilent) MSCV-RES-GFP (Addgene) and pMySc (Addgene) vectors. Fbxl1 was amplified from B6 thymocyte cDNA and cloned into the MSCV-RES-GFP (Addgene) vector. pCDNA3-myc-Fbxl1(Skp2), pGFP-E-Cdkn1b(p27), pCDNA3-DN-hCUL1-FLAG and pEGFP-C1-FLAG-Ku80 were purchased from Addgene. pCDNA3-myc-Fbxl12 and pMySc-Fbxl12 were used as a template to delete the F-box motif by PCR. Cdkn1b(K165R) was generated by site-directed mutagenesis with the Quick-Change Kit (Stratagene). Platinum-E Retroviral Packaging Cells were transfected with retroviral vectors. Retrovirus-containing medium was collected at 48 and 72 h post-transfection. For transduction, 2.5 × 10⁶ cells were incubated with 0.5 ml retrovirus-containing medium for 16 h and then replaced with fresh culture medium.

Flow cytometry and cell purification. Single-cell suspensions were prepared in Hank’s Balanced Salt Solution supplemented with 0.5% bovine serum albumin and 0.5% N2N cells. Cells were incubated with anti-FcR (2.4G2) for 10 min followed by fluorochrome-conjugated antibody staining for 50 min (4°C). For intracellular staining, after staining for surface antigens, cells were fixed in 2% paraformaldehyde (Polysciences) and permeabilized with 0.1% Triton X-100 (Sigma–Aldrich), then stained with 4’,6-diamidino-2-phenylindole (DAPI; Molecular Probes) and Ki-67 (BD Biosciences). Percentages of apoptotic cells were determined by Annexin V (BD Biosciences) staining according to the manufacturer’s instructions. Samples were analyzed on an LSR II or Fortessa flow cytometer (BD Biosciences). DNA thymocytes were purified by lineage marker negative selection using a magnetic bead/column system (MACS; Miltenyi Biotec). For DN3b cells, DN cells were further stained with CD27-PE, labelled with Anti-PE Microbeads and isolated by magnetic columns. The purification of γδT thymocytes, cells were first enriched by lineage marker (-TCRγδ) negative selection using a magnetic bead/column system (Miltenyi Biotec), followed by staining with TCRγδ-PE, positive selection with anti-PE mAb-conjugated microbeads and isolation by magnetic columns. Antibodies used for flow cytometry were as follows. The lineage marker (Lin) mixture for DN cells included the antibodies CD4 (GK1.5), CD8a (53-6.7), TCRβ (H57-597), TCRγδ (GL3), CD19 (1D3), B220 (RA3-6B2), Gr1 (RB6-8C5), Ter119, CD49b (D2S) and NK1.1 (PK136), all purchased from BD Bioscience. Other antibodies used for staining included CD4 (GK1.5; eBioscience), CD8 (53-6.7; eBioscience), CD24 (M1/69; BD Bioscience), CD25 (PC61; BD Bioscience), CD44 (S7; BD Bioscience), CD45.1 (A20; BD Bioscience), CD45.2 (A104; BD Bioscience), CD62L (MEL-14; BD Bioscience) and CD69 (H1.2F3; eBioscience).

Retroviral transductions of bone marrow-derived Rag2−/− progenitor T cells. Lineage (CD3, CD11b, CD11c, CD19, CD45R, CD161 or Ter119) PerCP-Cy5.5-negative CD117-APC-positive progenitors were isolated from the bone marrow of Rag2−/− mice, using flow cytometric cell sorting, and co-cultured with OP9-DL4 cells in OP9 media in the presence of IL-7 (5 ng/ml), SCI (50 ng/ml) and Flt3-L (1 ng/ml) for 7 d, to allow for T cell differentiation to the CD44+CD25− (DNS) stage. On day 7, differentiating T cells were cultured with GFP-, TCRγδ-SCF, TCRγδ-Flt3-L and IL-7. Transduced DN3 (CD45-Alexafluor-700) CD44+PerCP-Cy5.5+CD25−GFP+ cells were sorted by flow cytometry and cultured on OP9 or OP9-DL4 cells in the presence of the above-listed cytokines. Transduced CD45+GFP+ cells were harvested by flow cytometric cell sorting on days 1, 2 and 3 post-transduction.

Immunoprecipitation and immunoblot analysis. Cells were lysed in RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 1% sodium dodecyl sulfate) or NP-40 lysis buffer (50 mM Tris, 150 mM NaCl, 0.5% NP-40 and 1 mM EDTA) with protease inhibitor cocktail (Roche). Cell lysates were pre-cleared with Gammabind G Sepharose beads (GE Healthcare) for 20 min, then incubated with antibodies overnight, followed by a 2 h incubation with 30 μl Gammabind G Sepharose beads. Beads were washed three times with lysis buffer then boiled in LDS sample buffer (Invitrogen). For the in vivo ubiquitination assays, 293T cells were transfected with the indicated plasmids, including plasmid encoding Ub-HA. HA-Trap beads were used to isolate HA-tagged proteins from 4–12% Bis-Tris gels (Invitrogen), then transferred to PVDF membranes (Merck Millipore). The membranes were blocked for 1 h in phosphate buffered saline with Tween-20 containing 5% fat-free milk, then incubated with primary antibodies overnight followed by three washing steps and 1 h of incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies. Blots were developed with ECL (GE healthcare) and exposed to film (Kodak). The antibodies used were: HA (12CA5), β-actin (AC-74) and Flag (M2) (Sigma–Aldrich), Fbxl12 (ab96313) (Abcam); RORγt (NB2-24503) (Novus Biologicals); and Skp2 (H-435), Skp2 (A-2), CUL-1 (H213), c-Myc (A-14), c-Myc (9E10), p21 (C-19), p27 (F-8), p27 (C-19), Ku-86 (H-300), Fbxl12 (H-273), Goat anti-rabbit IgG-HRP (sc-2357), Goat anti-mouse IgG-HRP (sc-2032) and donkey anti-goat IgG-HRP (sc-2033) (Santa Cruz).

RNA isolation and real-time PCR. Total RNA was extracted from cells using TRIzol (Invitrogen) and reverse transcribed with the SuperScript First-Strand Synthesis system (Invitrogen). Transcripts were quantified with a Roche LightCycler 480 instrument. Gene expression levels were calculated and presented as expression relative to control genes.

Statistical analysis. All data are presented as means ± s.d. The unpaired, non-parametric Student’s t-test (Mann–Whitney t-test) was used for the statistical analysis. GraphPad Prism 7.0 was used for data analysis and presentation. P < 0.05 was considered statistically significant.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data that support the findings of this study are available from the corresponding author upon request.

References

51. Nakayama, K. et al. Targeted disruption of Skp2 results in accumulation of cyclin E and p27kip1, polyplody and centrosome overduplication. EMBO J. 19, 2069–2081 (2000).
52. Chien, W. M. et al. Genetic mosaics reveal both cell-autonomous and cell-nonautonomous function of murine p27kip1. Proc. Natl Acad. Sci. USA 103, 4122–4127 (2006).
53. Mohtashami, M., Shah, D. K., Kianizad, K., Awong, G. & Zuniga-Pflucker, J. C. Induction of T-cell development by Delta-like 4 expressing fibroblasts. Int. Immunol. 25, 601–611 (2013).
Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- n/a
- Confirmed

☐  The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement

☐  A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly

☐  The statistical test(s) used AND whether they are one- or two-sided

  *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*

☐  A description of all covariates tested

☐  A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons

☐  A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)

☐  For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted

  *Give P values as exact values whenever suitable.*

☐  For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings

☐  For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes

☐  Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

*Our web collection on [statistics for biologists](#) contains articles on many of the points above.*

Software and code

Policy information about [availability of computer code](#)

| Data collection | Flow cytometry: LSRII, LSRFortessa, BD ARIA II cell sorter, RT-PCR: LightCycler 480 |
|-----------------|-------------------------------------------------------------------------------------|
| Data analysis   | Flowjo 10, GraphPad Prism 7.0, BD FACSDiva 8.0                                      |

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The data that support the findings of this study are available from the corresponding author upon request.
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences  - Behavioural & social sciences  - Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-list.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

- Sample size: No statistical methods were used to predetermine sample sizes. Sample size was determined to ensure reproducibility of results and to achieve statistical significance, which was based on our and other similar previous studies.

- Data exclusions: No data were excluded.

- Replication: Reported experiments were repeated at least twice and were reliably reproducible.

- Randomization: No randomization was used as all experimental groups were based on genotypes of mice.

- Blinding: Blinding was not used since all data were collected and analyzed by software independently of biased intervention.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
|     | Antibodies            |
|     | Eukaryotic cell lines  |
|     | Palaeontology         |
|     | Animals and other organisms |
|     | Human research participants |
|     | Clinical data         |

### Methods

| n/a | Involved in the study |
|-----|-----------------------|
|     | ChiP-seq              |
|     | Flow cytometry        |
|     | MRI-based neuroimaging |

#### Antibodies

- CD4/BD Biosciences/553730/GK1.5/1:500
- CD8/BD Biosciences/550281/53-6.7/1:500
- TCRy/BD Biosciences/553177/ GL1/3/1:1000
- TCRβ/BD Biosciences/553171/ H57-597/1:1000
- CD19/BD Biosciences/561736/ 103/1/1:1000
- B220/BD Biosciences/561877/RA3-682/1:500
- G1.1/BD Biosciences/553126/RR6-8C5/1:1000
- CD49b/BD Biosciences/553126/DV5/1:1000
- NK1.1/BD Biosciences/557391/ PK136/1:1000
- CD45.1/BD Biosciences/561872/A20/1:500
- CD45.2/BD Biosciences/560695/A104/1:500
- CD62L/BD Biosciences/553151/ME1-14/1:1000
- CD25/BD Biosciences/557192/PC61/1:1000
- CD44/BD Biosciences/553135/57/1:500
- CD4/eBioScience/12-0041-42/GK1.5/1:500
- CD8/eBioScience/12-0081-82/53-6.7/1:500
- CD24/eBioScience/17-0242-82/M1/69/1:500
- CD68/eBioScience/H1.2F3/12-0891-82/1:500
- HA/ Sigma Aldrich/1583816001/12GAS/1:10000
- β-actin/Sigma Aldrich/A2228-200UL/AC-74/1:12000
- Flag/Sigma Aldrich/F1804-200UG/M2/1:10000
- Fbx12/Abcam/ab96831/1:2000
- Ku70/Abcam/ab83503/1:4000
- ROR gamma/Novus Biologicals/NBP2-24503/1:2000
Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)  HEK293T cells were obtained from ATCC. OP9, OP9-DL1 and OP9-DL4 cells are provided by Dr. Juan Carlos Zúñiga-Pflücker (University of Toronto). Platinum-E Retroviral Packaging Cell Line were brought from Cell Biolabs Inc.

Authentication  HEK293T cells were authenticated by ATCC. OP9, OP9-DL1 and OP9-DL4 cells were authenticated by Dr. Juan Carlos Zúñiga-Pflücker lab. Platinum E Retroviral Packaging Cell Line were authenticated by Cell Biolabs Inc. All cells were carefully check for morphology at the microscope after plating.

Mycoplasma contamination  All cell lines were tested Mycoplasma negative

Commonly misidentified lines (See ICCLAC register)  No commonly misidentified cell lines were used in this study

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals  C57BL/6 mice (CD45.1, CD45.2) and Rag2-/- mice were obtained from Taconic. Fbxl2 floxed mice have been generated as described in the method section. Fbxl1-/- mice were provided by Dr. Liang Zhu (Albert Einstein College of Medicine). Cdkn1b floxed mice were purchased from Jackson Laboratory. Both male and female mice at the age of 8-12 weeks were used in this study. Animal experiments were approved by the Animal Care and Use Committee of the National Institute of Child Health and Human Development.

Wild animals  No wild animals were used.

Field-collected samples  no field-collected samples were used.

Ethics oversight  All animal experiments were approved by the Animal Care and Use Committee of the National Institute of Child Health and Human Development.

Note that full information on the approval of the study protocol must also be provided in the manuscript.
Flow Cytometry

Plots

- Confirm that:
  - The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
  - The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
  - All plots are contour plots with outliers or pseudocolor plots.
  - A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Single-cell suspensions were prepared in HBSS supplemented with 0.5% BSA and 0.5% NaN3. Cells were incubated with anti-FcR (2.4G2) for 10min followed by fluorochrome-conjugated antibody staining for 50min (4 °C). For intracellular staining, after staining for surface antigens, cells were fixed in 2% paraformaldehyde (Polysciences) and permeabilized with 0.1% Triton X-100(Sigma-Aldrich), then stained with DAPI [Molecular Probes] and Ki-67 [BD Biosciences]. Percent of apoptotic cells was determined by Annexin V (BD Biosciences) staining according to the manufacturer's instructions.

Instrument

LSRII, LSRII-Fortessa, BD ARIA II cell sorter

Software

FACSDiva and FlowJo were used

Cell population abundance

The purity of the sorted cells was >95%, checked post sort

Gating strategy

Cells were gated by FSC-A/SSC-A to exclude debris, and FSC-H/SSC-W followed by SSC-H/SSC-W to exclude doublet cells. Detail gating strategies for specific population were described in figure/figure legends.

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.