Supplementary Figure 1 Analyses of hematopoietic compartments in cyclin $C^{D/D}$ mice. Ctrl and C-KO denote pI-pC treated cyclin $C^{F/F}$/Mx1-Cre^{-} and $C^{□/□}$/Mx1-Cre^{+} mice, respectively. (**a**) Western blot analysis of cyclin C in the indicated organs. BM, bone marrow. (**b**) FACS analysis of thymocytes. Cells were stained with anti-CD4 and -CD8 antibodies. (**c, d**) Mean proportion of the indicated thymocyte subsets (n=4 per genotype). DN, double-negative (please see Methods for staining). CD4+/CD8+ *P*=0.108, CD4+ *P* =0.093, CD8+ *P* =0.201, CD4-/CD8- *P* =0.0004, DN1 *P*=0.225, DN2 *P*=0.253, DN3 *P*=0.0007, DN3 to DN4 *P*=0.0013, DN4 *P*=0.052 (t-test). (**e**) Protein lysates prepared from thymocytes were analyzed by western blotting. Each lane corresponds to a separate animal of the indicated genotype. Note increased ICN1 levels in C-KO thymocytes. The levels of other regulators of thymocyte proliferation (cyclin D3, CDK6) were unchanged, or only slightly increased in C-KO. (**f**) Thymocytes were isolated from control and cyclin C-KO mice and analyzed by western blotting. Note that the levels of F-box protein Fbw7 (which controls ICN1 degradation) were not changed in C-KO cells. Moreover, the levels of other Fbw7 targets (Mcl1, cyclin E1, c-Myc) were not changed in C-KO cells. Also knockdown of cyclin C in human T-ALL cells had essentially no effect on the levels or half-life of these proteins (Supplementary Fig. 3c). Hence ablation of cyclin C selectively affects the stability of ICN1, but not of other Fbw7 substrates. (**g**) Numbers of red blood cells, white blood cells and platelets in peripheral blood (n=6 per group). Each dot corresponds to a separate animal, horizontal lines represent mean values. Red blood cells *P*=0.36, white blood cells *P*=0.40, platelets *P*=0.001 (t-test). (**h**) Mean total number of nucleated bone marrow cells (per one femur and tibia, n=5 per group). Error bars, S.D. *P*=0.21 (t-test). (**i**) Mean percentages of long-term hematopoietic stem cells (LT-HSC), short-term hematopoietic stem cells (ST-HSC), lymphoid-primed multipotent progenitors (LMPP) and common lymphoid progenitors (CLP) in bone marrow (n=4 per group). Error bars, S.D. LT-HSC *P*=0.958, ST-HSC *P*=0.603, LMPP *P*=0.993, CLP *P*=0.488 (t-test). (**j**), Lin^{-} bone marrow cells were cultured for 8 days on OP9-DL1 stroma. Cells were then stained for CD11b, CD19, CD4, CD8, CD25

and CD44, and analyzed by FACS. Shown are mean percentages of *in vitro* cultured cells displaying the indicated immunophenotype. Lin^- bone marrow was pooled from 4 mice per group. Data represent 2 technical replicates.

Supplementary Figure 2 Analyses of ICN1 stability in cyclin $C^{\Delta/\Delta}$ thymocytes. (**a**) Thymocytes were isolated from control (Ctrl, cyclin $C^{F/F}$/Mx1-Cre^-) and cyclin C-KO (cyclin $C^{\Delta/\Delta}$/Mx1-Cre) animals and treated with protein synthesis inhibitor cycloheximide (CHX, 100 μg/ml) for the indicated times. The levels of ICN1 were then examined by western blotting. (**b**) Thymocytes isolated from control and cyclin C-KO animals (genotypes as in **a**) were treated with a γ-secretase inhibitor Compound E (200 nM) for the indicated times. The levels of ICN1 were then examined by western blotting. (**c**) Thymocytes isolated from control and cyclin C-KO animals (genotypes as in **a**) were treated with proteasome inhibitors MG132 (10 μm), MG132 + lactacystin (MG132 + Lac, 10μm), or vehicle only (DMSO) for 6 hrs. Cell lysates were then prepared, and analyzed for ICN1 levels by western blotting.

Supplementary Figure 3 Analyses of human T-cell acute lymphoblastic leukemia (T-ALL) cells. (**a**) Human T-ALL MOLT-16 cells were transduced with viruses expressing anti-cyclin C shRNA or control shRNA (CTR). Shown are mean percentages of cells in the indicated cell cycle phases, error bars denote standard deviation of triplicate samples. G1 CTR to G1 cyclin C shRNA P=0.052, S CTR to S cyclin C shRNA P=0.993, G2/M CTR to G2/M cyclin C shRNA P=0.151 (t-test). (**b**) MOLT-16 cells were transduced with anti-cyclin C or control shRNAs (CTR). Cells were harvested and lysates probed with the indicated antibodies. (**c**) MOLT-16 cells were transduced with viruses expressing anti-cyclin C shRNAs (1, 2 or 3) or control shRNA (CTR). Cycloheximide (20 □g/ml) was added to the media and cells were harvested at the indicated time-points. Lysates were immunoblotted with the indicated antibodies. Note that knockdown of cyclin C strongly increased the half-life of ICN1, but it had no major

effect on half-lives of other Fbw7 targets (c-Myc, cyclin E, mTOR, Mcl1; see also Supplementary Fig. 1f). The half-life of ICN1 in cells depleted of cyclin C and in control cells was determined by densitometric analysis (ImageJ, NIH). (**d**) Combined knock-down of cyclin C and Fbw7. MOLT-16 cells were transduced with lentiviral vectors expressing shRNA against Fbw7 or cyclin C, or both. Lysates were immunoblotted using the indicated antibodies. Note that knockdown of cyclin C led to upregulation of ICN1 (compare ICN1, lanes 1 vs. 2). Knockdown of Fbw7 also increased ICN1 levels, as expected (compare ICN1, lanes 1 vs. 3). Importantly, cyclin C-knockdown did not further increase ICN1 levels in cells depleted of Fbw7 (ICN1, lanes 3 vs. 4), indicating that the two proteins operate in the same pathway. Fbw7-knockdown increased the levels of an Fbw7 target, cyclin E (compare lanes 1 vs. 3). (**e**) MOLT-16 cells were transduced with viruses encoding cyclin C, CDK8, or with empty vectors (EV). The levels of the indicated proteins were determined by immunoblotting. (**f**) MOLT-16 cells were transduced with viruses encoding HA-tagged cyclin C, CDK8, CDK3, CDK19 or empty vectors (EV). The levels of the indicated proteins were determined by immunoblotting. For detection of HA-tagged proteins, an anti-HA antibody was used. This antibody detects a background band that co-migrates with HA-CDK19 (star).

Supplementary Figure 4 Cyclin C-CDK8, C-CDK19 and C-CDK3 kinases regulate ICN1. (**a**) Protein sequences for human CDK8 (GI: 4502745), CDK19 (GI: 30387611), and CDK3 (GI: 4557439) were obtained from NCBI RefSeq. Multiple sequence alignment was performed with MAFFT (version 7) using the L-INS-i method. The resulting alignment was visualized using Jalview 2.8, highlighting identical amino acids between all three kinases in dark blue, and identical amino acids between two of the three kinases in light blue. (**b-d**) MOLT-16 cells were transduced with viruses encoding shRNAs against the indicated CDKs. The levels of ICN1 and CDKs were then determined by immunoblotting. (**e**) Validation of anti-CDK3 shRNAs. HeLa cells stably expressing HA-tagged CDK3 were used (to allow unequivocal detection of CDK3 with anti-HA antibody).

Cells were transduced with viruses expressing five different anti-CDK3 shRNAs (1-5). Knockdown of CDK3 was gauged by immunoblotting with an anti-HA antibody. shRNAs #2 and #3 were chosen and used for analyses shown in **d**.

(f) His-tagged CDK19, or cyclin C, or cyclin C plus His-tagged CDK19, were expressed in 293T cells. Complexes were immunoprecipitated using anti-His antibody (or with IgG, for control) and used in *in vitro* kinase reactions in the presence (+) or absence (-) of ICN1 as a substrate together with $\gamma[^{32}P]$ATP. As a positive control, ICN1 was incubated with recombinant cyclin C-CDK8. Upper panel: proteins analyzed by autoradiography to detect phosphorylated ICN1 (^{32}P-ICN1). Second panel: total GST-ICN1 protein detected by immunoblotting with anti-GST antibody (ICN1). Third and fourth panels: CDK19 and cyclin C detected using the indicated antibodies (anti-His antibody was used to detect tagged CDK19). (**g**) Whole cell lysates (Input) from experiment shown in **f**, immunoblotted with the indicated antibodies. Anti-His antibody was used to detect tagged CDK19. (**h**) HA-tagged CDK3, or cyclin C, or cyclin C plus HA-tagged CDK3 were expressed in 293T cells. Complexes were immunoprecipitated using anti-HA antibody (or with IgG, for control), and used in *in vitro* kinase reactions in the presence (+) or absence (-) of ICN1 as a substrate together with $\gamma[^{32}P]$ATP. As a positive control, ICN1 was incubated with recombinant cyclin C-CDK8. Upper panel: proteins analyzed by autoradiography to detect phosphorylated ICN1 (^{32}P-ICN1). Second panel: total GST-ICN1 proteins detected by Ponceau S staining. Third and fourth panels: CDK3 and cyclin C were detected using the indicated antibodies (anti-HA antibody was used to detect tagged CDK3). (**i**) Whole cell lysates (Input) from experiment shown in **h**, immunoblotted with the indicated antibodies. Anti-HA antibody was used to detect tagged CDK3.

Supplementary Figure 5 Cyclin C-CDK19 and C-CDK3 kinases phosphorylate T2512, S2514 and S2517 of ICN1. Cyclin C-CDK19 or cyclin C-CDK3 complexes purified from Sf9 cells were incubated with N-

EHPFLTPSPESPDQWFPK-C peptide, corresponding to aminoacids 2507 to 2521 of Notch1, as described in Methods. Separation of these Notch1 peptides by liquid chromatography after *in vitro* kinase reactions demonstrated three distinct peaks, each representing one of the three *in vivo* identified Notch1 phosphorylation sites (T2512, S2514 and S2517). Each of these identified phosphorylation sites could be confirmed by MS/MS analysis and sites localized using an Ascore localization score. Representative MS/MS scans are shown for both cyclin C-CDK19 and cyclin C-CDK3 experiments.

Supplementary Figure 6 Molecular analyses of cyclin C function. (**a, b**) Characterization of anti-phospho-Ser2517 ICN1 antibody. (**a**) Myc-tagged wild-type ICN1 (WT), or the indicated ICN1 mutants (TSS denotes T2512/S2514A/S2517A triple-mutant), or empty vectors (EV) were transfected into 293T cells. Cell lysates were probed with an anti-phospho-ICN1 antibody. (**b**) Upper panel: wild-type ICN1 (WT), or the indicated ICN1 mutants were expressed in 293T cells, immunoprecipitated with anti-Notch1 antibody, and immunoblotted with anti-phospho-ICN1 antibody. Lower panel: Notch1 was immunoprecipitated from I22 cells [a murine T-ALL line derived from a tumor induced by retrovirally-driven ICN1 (Pear et al. J. Exp. Med. 1996;183:2283)]. Immunoprecipitates were treated with lambda phosphatase (+) or left untreated (-), immunoblotted and probed with an anti-phosho-ICN1 antibody. Phosphatase treatment abolished immunoreactivity, as expected. (**c**) MOLT-16 cells were transduced with anti-cyclin C or control shRNA (CTR). ICN1 was immunoprecipitated (IP); normal IgG IP was used as a negative control. Immunoblots were probed with the indicated antibodies. Note reduced phosphorylation of endogenous ICN1, and reduced interaction of endogenous ICN1 and Fbw7 proteins in cyclin C-depleted cells. Input, immunoblotting of straight lysates. (**d**) Cyclin $C^{F/F}$ MEFs were transduced with Myc-tagged-ICN1. Cells were then treated with control adenovirus ($C^{F/F}$) or with an adenovirus encoding Cre ($C^{\Delta/\Delta}$). ICN1-Myc was immunoprecipitated and immunoblots were

probed using an antibody against Fbw7, or against Myc. Input, whole cell lysates. Note that the interaction between ICN1 and endogenous Fbw7 was strongly decreased upon ablation of cyclin C. (**e**) HeLa cells were transfected with HA-tagged Fbw7 along with an empty vector (EV), or Myc-tagged wild-type ICN1 (WT), or various ICN mutants containing alanine-substitutions within critical cyclin C-CDK-dependent phosphosites(TSS denotes T2512/S2514A/S2517A triple-mutant). ICN1 was immunoprecipitated using an anti-Myc antibody, and immunoblots were probed with an anti-HA antibody (to detect ICN1-bound Fbw7). Input, whole cell extracts. Note that alanine-substitutions inhibited the interaction of ICN1 with Fbw7 *in vivo.* (**f**) Cyclin $C^{F/F}$ MEF were transduced with Notch1 (WT-Notch1) or a Notch1 mutant (TSS-Notch1) in which the three critical cyclin C-CDK phosphorylation sites have been mutated to alanines (T2512A, S2514A S2517A). Cells were then transduced with control adenovirus ($C^{F/F}$), or an adenovirus encoding Cre ($C^{\Delta/\Delta}$). The levels of ICN1 were determined by western blotting. The middle portion of the gel was cut out and the images spliced together (dashed line). The expression of alanine-substituted mutant was strongly, as compared to the wild-type protein, and it was no longer affected by cyclin C-ablation. (**g**) HEK293 cells were transduced with anti-cyclin C or control shRNA (CTR). Cells were then transfected with Myc-tagged-ICN1 and His-tagged-ubiquitin. Ubiquitinated proteins were immunoprecipitated using Ni-NTA matrices and immunoblots were probed with an anti-Myc antibody to detect ubiquitinated ICN1. Input, whole cell lysates. Densitometric scanning of ubiquitinated ICN1 band intensities (normalized against input Myc-ICN1) revealed approximately 3-fold lower ubiquitination of ICN1 in cells depleted of cyclin C.

Supplementary Fig. 7 Analyses of cyclin C function in T-ALL. (**a**) HPC were isolated from pI-pC-treated $C^{F/F}$/Mx1-Cre^{-} (Ctrl), $C^{+/\Delta}$/Mx1-Cre (C-HET) and cyclin $C^{\Delta/\Delta}$/Mx1-Cre (C-KO) animals. pRB phosphorylation at Ser807/811 [a residue phosphorylated by cyclin C-CDK3 and D-CDK4 in human cells (Ren and Rollins,

Cell 2004;117:239; Zarkowska and Mittnacht, J. Biol. Chem. 1997;272:12738) was analyzed by immunoblotting. (**b**) HPC were isolated from mice of the indicated genotypes (as in **a**) and left untreated (Lin-BM), or cultured for 3 days on OP9-DL1 stroma (Lin-BM/OP9-DL1). Alternatively, HPC were transduced with Notch1-P12 (Lin-BM/Notch1-P12) or ICN1 (Lin-BM/ICN1). Cells were pulsed with BrdU and analyzed by FACS. Shown is the mean percentage of BrdU-positive cells; error bars, S.D. HPC (Lin^-) were pooled from 4 mice per group. Data represent 2 biological replicates. (**c**) Hematoxylin and eosin stained sections of organs collected at 4.5 wks post bone marrow transplantation, from mice which received ICN1-transduced control and C-KO HPC. Note the presence of infiltrating tumor cells (arrows) in recipients of C-KO cells, but not control cells. Scale bar, 500μm. (**d**) Organs were collected from recipients of ICN1-transduced Ctrl and C-KO HPC. Animals were injected with BrdU, GFP^+ tumor cells were sorted, and the percentage of BrdU-positive cells was evaluated by FACS. Each dot corresponds to a separate animal. Horizontal lines denote mean values, error bars, SD. *P*=0.2452 for bone marrow, *P*=0.0114 for spleen, *P*=0.1554 for thymus (unpaired t-test). (**e**) HPC from Ctrl and C-KO mice (genotypes as in **a**) were transduced with Notch1-P12 and GFP, and injected into recipient mice. The percentage of leukemic cells (GFP^+,$CD4/CD8^+$) in peripheral blood was evaluated by FACS after 7 weeks. Each dot corresponds to a separate animal, horizontal lines denote mean values. p=0.0171 (Mann-Whitney test). (**f**) Similar experiment as in Fig. 6h in the main text, except that control and cyclin C-KO HPC were transduced with a mutant version of Notch1 (ΔEGFΔLNR). This mutant lacks C-terminal aminoacids 2473-2556, which harbor the three essential cyclin C-CDK phosphorylation sites. Left: tumor incidence, n=10 per group. Right: Kaplan-Meier survival analysis, n=10 per group. (**g**) CDK19 expression was analyzed using gene expression microarrays on a subset of primary T-ALL patient samples shown in the main text in Fig. 7a.

Supplementary Fig. 8 Scans of western blots.

Supplementary Table 1 Proteomic analysis of *in vitro* and *in vivo* Notch 1 phosphorylation sites.

Supplementary Table 2 Mass spectrometry detection of cyclin C-CDK3 and cyclin C-CDK19 *in vitro* phosphorylation of a synthetic Notch 1 peptide.

Supplementary Table 3 Mass spectrometry detection of cyclin C-CDK8 *in vitro* phosphorylation of synthetic Notch1 peptides mimicking patient-derived mutations.