

Loss of mTOR complex 1 induces developmental blockage in early T-lymphopoiesis and eradicates T-cell acute lymphoblastic leukemia cells

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mTOR is an evolutionarily conserved kinase that plays a critical role in sensing and responding to environmental determinants. Recent studies have shown that fine-tuning of the activity of mTOR complexes contributes to organogenesis and tumorigenesis. Although rapamycin, an allosteric mTOR inhibitor, is an effective immunosuppressant, the precise roles of mTOR complexes in early T-cell development remain unclear. Here we show that mTORC1 plays a critical role in the development of both early T-cell progenitors and leukemia. Deletion of *Raptor*, an essential component of mTORC1, produced defects in the earliest development of T-cell progenitors in vivo and in vitro. Deficiency of *Raptor* resulted in cell cycle abnormalities in early T-cell progenitors that were associated with instability of the Cyclin D2/D3-CDK6 complexes; deficiency of *Rictor*, an mTORC2 component, did not have the same effect, indicating that mTORC1 and -2 control T-cell development in different ways. In a model of myeloproliferative neoplasm and T-cell acute lymphoblastic leukemia (T-ALL) evoked by *Kras* activation, *Raptor* deficiency dramatically inhibited the cell cycle in oncogenic *Kras*-expressing T-cell progenitors, but not myeloid progenitors, and specifically prevented the development of T-ALL. Although rapamycin treatment significantly prolonged the survival of recipient mice bearing T-ALL cells, rapamycin-insensitive leukemia cells continued to propagate in vivo. In contrast, *Raptor* deficiency in the T-ALL model resulted in cell cycle arrest and efficient eradication of leukemia. Thus, understanding the cell-context-dependent role of mTORC1 illustrates the potential importance of mTOR signals as therapeutic targets.

mTOR is a serine/threonine kinase that has a central role in the regulation of cell growth and cell metabolism and forms two functionally different complexes, named mTORC1 and mTORC2 (1). The Raptor subunit is specific to the mTORC1 complex, and Rictor is specific to mTORC2. One of the major upstream signal transduction pathways of mTORC1 is the phosphatidylinositol-3 kinase (PI3K)-AKT pathway. AKT activates mTORC1 via PRAS40 and the tuberous sclerosis 1/2 (TSC1/2)-Rheb pathway. The TSC1/2 complex is an established mTORC1 suppressor, and its protein destabilization via extracellular-signal-regulated kinase (ERK) activates mTORC1 (2). Because the GTP-bound form of Ras interacts with and activates PI3K and ERK, Ras is also an activator of mTORC1 (3).

Abnormalities of mTOR signals are frequently detected in patients with one of several types of leukemia (4, 5). In particular, alterations in PTEN, PI3K, or AKT frequently occur in patients with T-cell acute lymphoblastic leukemia (T-ALL) (6). In a mouse model, deletion of *Pten* during hematopoiesis demonstrated that *Pten* is critical for suppressing the development of

leukemia (7–9). Furthermore, studies using *Raptor*- or *Rictor*-deficient mice revealed that activation of mTORC1 or -2 is required for the leukemogenesis evoked by *Pten* loss (10, 11). However, the involvement of mTORC1 in leukemogenesis associated with other oncogenic signals, such as Ras, is not well understood. More importantly, it has remained unclear whether mTORC1 inactivation would eradicate T-ALL.

Rapamycin is a potent immunosuppressant that induces severe thymic atrophy in rodents. However, a study of conditional deletion of *Rheb*, which encodes an mTORC1 activator, or of *mTOR* with a *Cd4-Cre* transgene showed that mTORC1 inactivation does not result in apparent thymic phenotypes under steady-state conditions (12), leading to the possibility that rapamycin may affect T-cell development in an mTORC1-independent manner. In addition, it has been reported that 4E-BP1 is a rapamycin-insensitive mTORC1 substrate, suggesting that rapamycin treatment does not necessarily represent mTORC1 inactivation (13).

Significance

mTOR, a kinase that senses and responds to nutrients, plays critical roles in organogenesis and tumorigenesis. Although mTOR inhibitors have been developed as immunosuppressants and anticancer drugs, it has remained controversial whether such medications contribute to cancer eradication. In addition, mTOR inhibition by chemical inhibitors is complicated because it may not produce predictable inhibition of the mTOR complexes mTORC1 and mTORC2. By using a genetic approach, our study clearly demonstrates that mTORC1, but not mTORC2, is essential for cell cycling of early T-cell progenitors. More importantly, we reveal that loss of mTORC1 efficiently eradicates T-cell acute lymphoblastic leukemia cells, but not myeloid leukemia. Thus, understanding the cell-context-dependent role of mTOR illustrates the potential importance of mTOR signals as therapeutic targets.

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Thus, the precise roles of mTOR complexes in T-cell development remain unclear.

In this study, we focused on the role of mTOR in T-cell development. Our data clearly show that mTORC1, but not mTORC2, is essential for cell cycling of the earliest T-cell progenitors, but not myeloid progenitors. In addition, we found that mTORC1 inactivation effectively prevented the induction of T-ALL, but not myeloproliferative neoplasm (MPN), induced by oncogenic *Kras*, indicating that mTORC1 is specifically essential for T-cell development and leukemogenesis. Importantly, we revealed that inactivation of mTORC1 by *Raptor* deficiency efficiently eradicates Notch-driven T-ALL in vivo. Thus, dissection of mTOR signals in vivo should suggest therapeutic approaches that will successfully eradicate many types of cancer.

Results

Raptor Deficiency Impairs Development of Early T-Cell Progenitors in Vivo. To understand the physiological role of mTORC1 in T-cell development, we evaluated the effects of mTORC1 inhibition by rapamycin treatment or the genetic deletion of the *Raptor* gene. As previously reported (14), rapamycin treatment resulted in apparent atrophy of the thymus in wild-type mice and hypophosphorylation of S6, a representative mTORC1 downstream target, in thymocytes (Fig. 1A and Fig. S1A–G). Higher doses of rapamycin did not show a significant change of effects on thymic atrophy and the proportion of thymocytes (Fig. S1A–F). We analyzed thymuses from tamoxifen (TAM)-induced *Raptor*-deficient

mice (*Raptor*^{fl/fl}; *Rosa26-CreER*^{T2}), in which *Raptor* is efficiently deleted in all tissues, including hematopoietic cells, by 2 wk after TAM treatment. We found that, similar to rapamycin treatment, *Raptor* deficiency resulted in thymic atrophy (Fig. 1A and Fig. S1H). Flow cytometric analysis revealed that *Raptor* deficiency dramatically inhibited the development of CD4/CD8 double-positive (DP) cells (Fig. 1B). Although the degree of T-cell development in *Raptor*-deficient mice varied highly among individuals (Fig. 1B), three of nine mice showed a particularly severe reduction of DP cells (Fig. 1B). Rapamycin treatment produced similar defects, but to a lesser extent (Fig. 1B and Fig. S1C and F). Next, to examine the effects of T-cell-specific *Raptor* deficiency, we evaluated thymic phenotypes in mice lacking *Raptor* in T cells (*Raptor*^{fl/fl}; *Lck-Cre*, Fig. S2A). However, we unexpectedly did not find any significant reduction of thymocytes (Fig. S2B) or apparent blockage of cell differentiation in thymocytes (Fig. S2C). To investigate the frequency of gene deletion in the *Raptor*^{fl/fl}; *Lck-Cre* mice, we generated *Raptor*^{fl/fl}; *Lck-Cre* mice carrying a red fluorescent protein (RFP) reporter allele, in which Cre recombinase activity can be monitored by the expression of RFP protein (15). We found that only 3.7% of CD4/CD8 double-negative (DN) 1 (CD44⁺CD25⁻) cells and 29.4% of DN3 (CD44⁺CD25⁺) cells expressed RFP protein (Fig. S2D), indicating that Cre recombinase was not fully expressed or activated at stages DN3 in our experimental condition. Furthermore, we found that *Raptor* protein was not reduced even in the RFP-positive (Cre-expressing) DN3 (Fig. S2E), presumably due to the long half-life of the *Raptor* protein. Accordingly, the phosphorylation level of 4E-BP1 was also comparable to that of the control (Fig. S2F) in DN3 cells. In contrast, CD4/CD8 DP cells showed clear induction of RFP expression (Fig. S2D), reduced *Raptor* protein (Fig. 2E), and reduced phosphorylation of 4E-BP1 (Fig. S2F), indicating that *Raptor* is dispensable for T-cell development at the DP stage.

Next, we focused on phenotypes in thymic DN cells of *Raptor*^{fl/fl}; *Rosa26-CreER*^{T2} mice. We found that S6 exhibited relatively high levels of phosphorylation in early T-cell progenitors, including DN1 and DN2 (CD44⁺CD25⁺), suggesting that activation of mTORC1 may be crucial for the development of early T-cell progenitors (Fig. 1C). Rapamycin remarkably increased the proportion of DN3 cells and decreased the proportion of DP cells (Fig. 1B and D and Fig. S1C, D, and F), indicating that rapamycin mainly blocked differentiation from DN3 to DP in vivo. *Raptor* deficiency resulted in an increased proportion of DN1, whereas the proportions of DN2 and DN3 were reduced (Fig. 1D). We confirmed that the *Raptor* gene was completely deleted in DN3 cells from thymus (Fig. S2G), even in cases that showed milder phenotypes. Although the absolute number of *Raptor*-deficient DN1 in thymus varied among samples, there was no significant difference compared with control (Fig. S2H and I). In contrast, the absolute number of DN2 and DN3 was dramatically reduced in *Raptor*-deficient mice (Fig. S2J and K). These data suggest that development of early T-cell progenitors, particularly at DN2, may be impaired by *Raptor* deficiency in vivo.

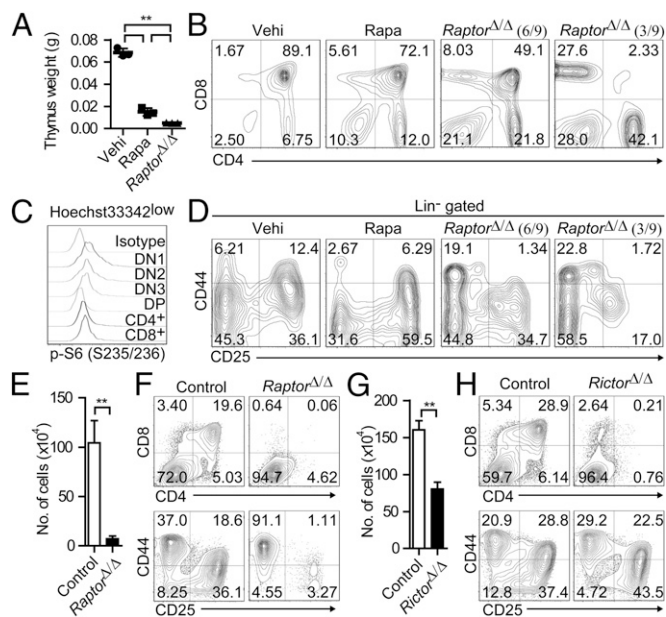


Fig. 1. Abnormal T-cell development by mTORC1 inactivation. (A) Thymus weight of mice treated with vehicle (Vehi) or rapamycin (Rapa) for 2 wk and *Raptor*^{fl/fl}; *Rosa26-CreER*^{T2}+TAM (*Raptor*^{ΔΔ}) mice at 2 wk post-TAM ($n = 3$ mice/group). (B) Flow cytometric analyses of differentiated T cells in thymuses from vehicle-treated, rapamycin-treated, and *Raptor*^{ΔΔ} mice. Representative data from at least three individual experiments are shown. (C) Phosphorylation level of ribosomal protein S6 in the indicated T-cell subpopulations. Alexa488-conjugated isotype IgG (Iso) was used as a negative control. (D) Flow cytometric analyses of T-cell progenitors in thymuses from vehicle-treated, rapamycin-treated, and *Raptor*^{ΔΔ} mice. Representative data from at least three individual experiments are shown. (E–H) *Raptor*^{fl/fl}; *CreER* (E and G) or *Rictor*^{fl/fl}; *CreER* (F and H) LSK cells were cocultivated with Tst-4/ DLL1 stromal cells for 16 d in the presence of 4-OHT in vitro. (E and F) Number of cells ($n = 4$ experiments). (G and H) Representative data from flow-cytometric analyses. (Lower) Cells are DN (CD4⁻CD8⁻) gated cells. For A, E, and G, $**P < 0.01$ (Student *t* test).

mTORC1, but Not mTORC2, Plays a Critical Role in the Development of the Earliest T-Cell Progenitors in Vitro. To investigate how mTORC1 controls the development of early T-cell progenitors and whether the effect of *Raptor* deficiency is cell-intrinsic, we evaluated the development of T cells in vitro. To do so, we cultured Lineage⁻Sca-1⁺c-KIT⁺ (LSK) cells, which are hematopoietic stem and progenitor cells, from bone marrow (BM) of adult *Raptor*^{fl/fl}; *Rosa26-CreER*^{T2} mice, on DLL1 (Notch1 ligand)-expressing stromal cells to evaluate the proliferation and differentiation of T cells (16). When we deleted *Raptor* by adding 4-hydroxytamoxifen (4-OHT) to this culture, T-cell proliferation was dramatically reduced (Fig. 1E and F and Fig. S3A). Consistent with the phenotypes observed in vivo (Fig. 1B and D), *Raptor* deficiency enriched the DN1 fraction and resulted in a remarkable

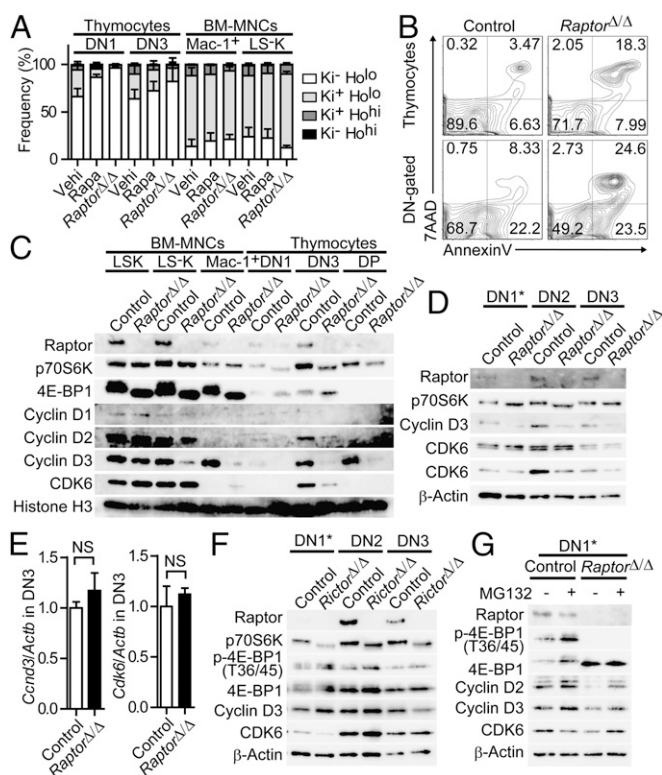


Fig. 2. Cell cycle regulation of T-cell progenitors by mTORC1 via the Cyclin D2/D3-CDK6 complex. (A) Cell cycle. Cells from the indicated hematopoietic subpopulations of vehicle-treated (Vehi), rapamycin-treated (Rapa), and *Raptor*^{Δ/Δ} mice were analyzed with *K_i-67*/Hoechst33342 staining (*n* = 3). Lineage⁺Sca-1⁺c-KIT⁺ (LSK) cells were analyzed as BM hematopoietic progenitor cells. (B) Apoptosis rate. All thymocytes (Upper) and DN thymocytes (Lower) were evaluated by using AnnexinV/7AAD staining. AnnexinV⁺7AAD⁺ cells were considered to be apoptotic. Data shown are representative of two independent experiments. (C) Protein expression of Cyclin D1, D2, D3, CDK6, and mTORC1-related molecules in the indicated subpopulations of hematopoietic cells from *Raptor*-deficient mice at 2 wk post-TAM. (D) Cyclin D3 and CDK6 protein in *Raptor*-deficient developing T cells in vitro. (E) mRNA expression of Cyclin D3 (*Cnd3*) and CDK6 (*Cdk6*) in DN3 thymocytes from *Raptor*-deficient mice at 2 wk post-TAM (*n* = 3). The values were normalized to the expression of β-actin (*Actb*). (F) Cyclin D3 and CDK6 protein in *Raptor*-deficient developing T cells in vitro. (G) Protein amount of Cyclin D2, D3, and CDK6 in the absence or presence of the proteasome inhibitor MG-132. DN1* cells from in vitro culture were analyzed 2 h after MG-132 administration. NS, not significant.

failure of DN2 and DN3 cells to develop, indicating that mTORC1 inactivation results in failure of development from DN1 to DN2. To confirm that inactivation of mTOR kinase itself would produce the same defective phenotypes, we generated *mTOR*^{fl/fl}; *Rosa26-CreER*^{T2} mice (Fig. S4 A–D) and evaluated the effect of mTOR deficiency on T-cell development. As expected, mTOR deficiency also suppressed the proliferation and development of T cells in vitro (Fig. S3 B and C), as we had observed in *Raptor*-deficient cells. Furthermore, to investigate the role of mTORC2 on T-cell development in this experimental condition, we generated *Rictor*^{fl/fl}; *Rosa26-CreER*^{T2} mice. *Rictor*-deficient cells showed comparable proliferative capacity to the control (Fig. S3 D and E) after 11 d in culture, and differentiation of stages DN2 and DN3 was normal. After culture for a longer period (16 d), *Rictor* deficiency eventually resulted in a reduction of the total cell number, and DP cells failed to develop (Fig. 1 G and H), indicating that mTORC2 plays a critical role in the development of DP cells. These results clearly demonstrate that mTORC1 and mTORC2 control T-cell development in a different manner and

that mTORC1 plays a critical role in the earliest development of T-cell progenitors.

mTORC1 Activity Is Required for Cell Cycling of the Earliest T-Cell Progenitors. To investigate how mTORC1 controls development of the early T-cell progenitors, we evaluated the expression profiles of selected genes in T-cell development. Several genes that were up-regulated or down-regulated during T-cell development were regulated in the same manner in *Raptor*-deficient thymocytes (Fig. S5 A–G), suggesting that *Raptor* deficiency does not directly affect the differentiation program. Consistent with these data, DN cells from *Raptor*-deficient mice showed T-cell receptor β (TCRβ) gene rearrangement (Fig. S5H). Next, we examined the cell cycle status of *Raptor*-deficient T-cell progenitors by using *K_i-67* and Hoechst33342 staining. *Raptor*-deficient DN1 cells showed a dramatic decrease of the S/G2/M phases and an increase of the G0/G1 phases (Fig. 2A), indicating that *Raptor* deficiency caused a defect in the G1/S transition in early T-cell progenitors in thymus. Rapamycin also inhibited the cell cycle of DN1, but to a lesser extent than *Raptor* deficiency. Although *Raptor* deficiency significantly increased the number of 7AAD⁺ dead cells, no remarkable increase of apoptotic cells (7AAD⁺AnnexinV⁺) was seen (Fig. 2B). Consistent with this result, amounts of survival-related (Bcl-xL and Bcl-2) and apoptosis-related (cleaved PARP and cleaved Caspase-9) proteins were not affected by *Raptor* deficiency in T-cell progenitors (Fig. S6A). These data suggest that mTORC1 inactivation may primarily cause a failure of the cell cycle, resulting in cell death. Thus, mTORC1 activation is essential for T-cell development in thymus, presumably because it supports proliferation of the earliest T-cell progenitors.

Cyclin D2/D3-CDK6 Protein Complex Is Stabilized by mTORC1 Activity. To find mTORC1 targets responsible for phenotypes of *Raptor*-deficient early T-cell progenitors, particularly in DN1, we performed Western blotting. We found that *Raptor* deficiency induced a band-shift of p70S6K and 4E-BP1 proteins in all hematopoietic subpopulations, including DN1 cells (Fig. 2C), and confirmed that phosphorylation of 4E-BP1 was reduced (Fig. S6A), indicating that mTORC1 was completely inactivated. The Cyclin D3-CDK6 complex has an indispensable role in the cell cycle of normal T-cell progenitors and T-ALL cells (17, 18). Because we observed the apparent cell cycle arrest only in T-cell progenitors, we evaluated the protein expression of Cyclin D1, D2, and D3 and CDK6 in several subpopulations of *Raptor*-deficient hematopoietic cells (Fig. 2C). Cyclin D2 and D3 expression was dramatically reduced in *Raptor*-deficient cells in differentiated hematopoietic subpopulations. Consistent with a previous report that CDK6 expression is regulated by the Notch-AKT pathway (17), we found that the CDK6 protein level was increased in DN3 cells in association with the up-regulation of Notch target genes (Tcf7, Il2ra, Fig. S5 D and E). Although *Raptor* deficiency did not abrogate the up-regulation of CDK6 expression in DN3, the protein level of CDK6 in the *Raptor*-deficient T-cell progenitors appeared to be lower than in the control (Fig. 2C). In contrast, CDK6 expression was increased in *Raptor*-deficient myeloid-lineage cells. *Raptor* deficiency also strongly reduced the protein level of Cyclin D3 in the in vitro culture system (Fig. 2D). Although the CDK6 protein level varied among samples, it was decreased to a lesser extent than Cyclin D3 (Fig. 2D). The mRNA transcriptional levels of Cyclin D3 and CDK6 were not affected by *Raptor* deficiency in DN3 cells (Fig. 2E). Protein levels of Cyclin D3 and CDK6 were not affected by *Rictor* deficiency (Fig. 2F). These data suggest that mTORC1 activity may control the Cyclin D2/D3-CDK6 complex via posttranscriptional mechanisms. Because the amount of Cyclin D3 protein is regulated by ubiquitin-mediated proteolysis via PI3K activity (19), we next examined the roles of mTORC1 in proteolysis-mediated regulation of Cyclin D2/D3 in T cells. When we treated T cells with a proteasome

inhibitor, MG-132, in vitro, the reduction of Cyclin D2/D3 and CDK6 by mTORC1 inactivation was reversed in both DN1 cells and DN3 cells (Fig. 2G and Fig. S6B). These data suggest that mTORC1 controls the cell cycle of early T-cell progenitors, including DN1 and DN3, by stabilizing the Cyclin D2/D3-CDK6 complex.

Inactivation of mTORC1 Prevents Oncogenic Kras-Induced T-ALL Development. A mouse model with an oncogenic Kras mutation (*Kras*^{G12D}) develops MPN, followed by T-ALL (20–23). We evaluated the effect of *Raptor* deficiency on oncogenic Kras-driven hematopoiesis, particularly on the development of leukemia. To do so, we transplanted *Raptor*^{fl/fl}; *Rosa26-CreER*^{T2}, *LSL-Kras*^{G12D}; *Rosa26-CreER*^{T2}, or *Raptor*^{fl/fl}; *LSL-Kras*^{G12D}; *Rosa26-CreER*^{T2} BM cells as tester cells (CD45.2) and equal numbers of competitor (CD45.1) wild-type BM cells into recipient mice (CD45.1) and administered TAM 4 wk after transplantation. At 3 wk post-TAM, we examined the cell lineages in the thymus and BM of recipient mice bearing *Raptor*^{Δ/Δ}, *Kras*^{G12D}, and *Raptor*^{Δ/Δ} *Kras*^{G12D} cells (Fig. 3A and B). The proportion of *Raptor*^{Δ/Δ} tester cells, compared with competitor cells, was dramatically reduced in thymus 3 wk after TAM administration, whereas it was de-

creased only slightly in BM. *Raptor* deficiency dramatically inhibited the proliferative effects of *Kras*^{G12D} on the cell competition in thymus. mTORC1-independent phosphorylation of S6 was observed in Mac-1⁺ myeloid cells (Fig. 3C). In contrast, both S6 and 4E-BP1 were hypophosphorylated in *Raptor*-deficient T cells, indicating that the phosphorylation was completely controlled by mTORC1 in T cells (Fig. 3C). *Raptor* deficiency inhibited the cell cycle in oncogenic Kras-expressing T-cell progenitor cells, but not in Mac-1⁺ myeloid cells (Fig. 3D). When we analyzed the *Raptor*^{Δ/Δ} *Kras*^{G12D} mice at 2 wk post-TAM, we found that the combination of *Raptor* deficiency and Kras activation resulted in an increase of the number of monocytes and granulocyte-macrophage progenitors (GMPs) (Fig. S7A–E). This finding was in contrast to the severe thymic atrophy that we observed in these mice (Fig. S2H–K), highlighting the interesting difference in the roles of Raptor between myeloid and lymphoid cells. Consistent with the hypophosphorylation of 4E-BP1, rates of new protein synthesis were significantly reduced in cycling *Raptor*-deficient progenitors (Fig. S7F). These data indicate that the impact of mTORC1 deficiency on cell cycle status varies substantially depending on the cell context and that Raptor is critical for the development and proliferation of T cells, even when the T cells are oncogenically activated.

To evaluate the effect of mTORC1 inactivation on the leukemogenesis induced by oncogenic Kras, we performed a long-term observation of hematopoiesis in mice bearing mutant cells. Mice bearing control or *Raptor*^{Δ/Δ} cells survived to the end of the experiment (Fig. 3E). Most of the mice with *Kras*^{G12D} cells died by 83 d after TAM treatment (Fig. 3E), with an associated overt increase of white blood cells (WBC) (Fig. 3F). The increase of WBCs was due to the propagation of CD4⁺CD8⁺ cells in some cases, indicating that the mice had developed T-ALL (Fig. 3G). In contrast, mice with *Raptor*^{Δ/Δ} *Kras*^{G12D} cells died by 131 d after TAM treatment (Fig. 3E); three of seven *Raptor*^{Δ/Δ} *Kras*^{G12D} mice showed an elevated WBC count by 16 wk post-TAM (Fig. 3F), but others did not. Strikingly, none of the *Raptor*^{Δ/Δ} *Kras*^{G12D} mice showed an increase of CD4⁺CD8⁺ cells in peripheral blood (PB) (Fig. 3G). All *Raptor*^{Δ/Δ} *Kras*^{G12D} mice with elevated WBC counts showed an increase of the Mac-1⁺Gr-1⁺ myeloid-lineage cell population in PB (Fig. 3G and Fig. S7G), suggesting that the cause of death of these mice may have been MPN. Thus, inhibition of mTORC1 dramatically suppressed T-ALL development in response to oncogenic Kras expression.

mTORC1 Inactivation Efficiently Eradicates Notch-Driven T-ALL. To investigate the effects of mTORC1 inhibition on the proliferation of T-ALL in vivo, we transplanted cells from thymuses of recipient mice that showed obvious signs of T-ALL development into new recipients (Fig. S8A–C). Although the survival of recipient mice bearing Kras-evoked T-ALL cells was significantly prolonged by rapamycin treatment (Fig. 4A and Fig. S8D), T-ALL cells continued to propagate in BM and spleen of rapamycin-treated mice (Fig. S8E), and the mice eventually died, indicating that rapamycin-insensitive T-ALL can survive and proliferate in vivo. Hence, we investigated the impact of complete mTORC1 inactivation on eradication of T-ALL cells in vivo. To do so, we generated a T-ALL model in which an active *Notch1* (NICD) gene, along with GFP as a marker, is retrovirally introduced into BM cells from *Raptor*^{fl/fl}; *Rosa26-CreER*^{T2} mice (Fig. S9A). These BM cells are then transplanted into lethally irradiated mice. We also evaluated the effect on the behavior of T-ALL of hyperactivation of mTORC1 by deletion of *Tsc1*, which is a negative regulator of mTORC1. *Tsc1* deficiency significantly shortened the survival of T-ALL mice, whereas *Raptor* deficiency completely suppressed death in the T-ALL mice (Fig. 4B). As expected, *Tsc1* deficiency enhanced propagation of GFP⁺ T-ALL cells in the PB (Fig. 4C). In contrast, *Raptor* deficiency dramatically decreased propagation of T-ALL cells in the PB (Fig. 4C). Furthermore, we

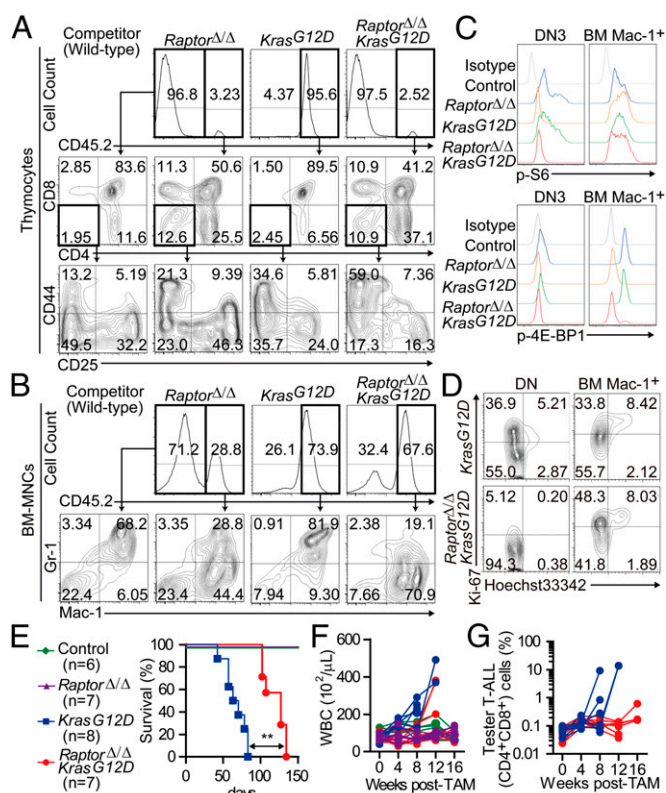


Fig. 3. *Raptor* deficiency suppresses the development of oncogenic Kras-induced T-ALL. (A and B) Flow cytometric analyses of thymocytes (A) and BM-mononuclear cells (MNCs) (B) from recipient mice competitively reconstituted with whole BM cells from the indicated mice (CD45.2) and wild-type competitor whole BM cells (CD45.1). Samples were collected at 3 wk post-TAM. Representative data from three individual experiments are shown. (C) Phosphorylation levels of S6 (Upper) and 4E-BP1 (Lower) in DN3 T-cell (Left) and BM Mac-1⁺ myeloid (Right) tester (CD45.2⁺) cells. (D) Cell cycle of DN thymocytes (Left) and BM Mac-1⁺ myeloids (Right) from *Kras*^{G12D} and *Raptor*^{Δ/Δ} *Kras*^{G12D} mice. The cell cycle status was evaluated by using K₇-67/Hoechst33342 staining. (E) Survival of recipient mice competitively reconstituted with hematopoietic cells from mice of the indicated genotypes. TAM was administered from 8 wk after transplantation. ***P* < 0.0001, log-rank test. The color key applies to E–G. (F) Number of WBC in PB (*n* = 6–8). Each line represents data from an individual mouse. (G) Frequency of CD4⁺CD8⁺ T-ALL cells in PB (*n* = 7–8).

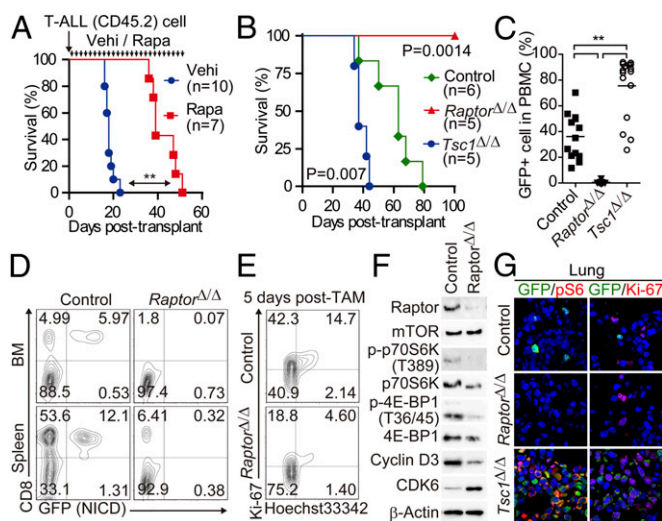


Fig. 4. Efficient eradication of Notch-driven T-ALL by *Raptor* deficiency. (A) Survival of recipient mice bearing *Kras*^{G12D} T-ALL cells after vehicle or rapamycin treatment. $^{**}P < 0.0001$, log-rank test. (B) Survival of recipient mice bearing NICD-GFP-transduced cells. P values, log-rank test. (C) Frequency of GFP⁺ PB-MNCs from recipient mice bearing NICD-GFP-transduced cells at 28 d after transplantation ($n = 13$ – 16). Horizontal lines show the mean. (D) Flow cytometric analyses of BM-MNCs and splenic MNCs from recipient mice bearing NICD-GFP-transduced cells at 28 d after transplantation. Representative data from two independent experiments are shown. (E) Cell cycle of *Raptor*-deficient GFP⁺ T-ALL cells. Cells were collected from splenic MNCs from T-ALL mice at 5 d post-TAM. The cell cycle status was evaluated by using *Ki-67*/Hoechst33342 staining. (F) Protein expression of Cyclin D3, CDK6, and mTORC1-related molecules in *Raptor*-deficient GFP⁺ T-ALL cells from T-ALL mice at 5 d post-TAM. (G) Expression of p-S6(S235/236) and *Ki-67* in NICD-GFP-transduced cells in lung. Nuclei were counterstained with DAPI (blue). For C, $^{**}P < 0.01$ (Student *t* test).

found that, unlike rapamycin treatment, *Raptor* deficiency efficiently eradicated T-ALL in BM and spleen (Fig. 4D). The cell cycle was apparently inhibited in *Raptor*-deficient T-ALL cells (Fig. 4E). The expression of Cyclin D2 and D3, but not CDK6, was strongly suppressed in *Raptor*-deficient T-ALL cells (Fig. 4F and Fig. S9B). The expression levels of apoptosis-related molecules were not remarkably affected by *Raptor* deficiency in T-ALL cells (Fig. S9B). These data suggest that mTORC1 strictly controls Cyclin D2 and D3 protein levels in all types of cells, whereas CDK6 protein may be controlled by mTORC1 in a cell-context-dependent manner. In control T-ALL mice, leukemia cells disseminated and proliferated in several nonhematopoietic organs, including liver and lung, but we did not find any dissemination of *Raptor*-deficient leukemia cells (Fig. 4G and Fig. S9C). In contrast, *Tsc1* deficiency exacerbated the dissemination and proliferation of T-ALL cells in nonhematopoietic organs (Fig. 4G and Fig. S9C). Furthermore, we investigated whether *Raptor* deficiency can deplete leukemia cells in a more aggressive T-ALL model. It was recently reported that oncogenic mutations of NRAS and KRAS cause early T-cell precursor ALL, an aggressive subtype of T-ALL (24). Because we observed that T-ALL cells driven by oncogenic *Kras* and associated with Notch1 mutations were rapamycin-insensitive (Fig. 4A and Fig. S8E), we combined Notch-driven T-ALL with *Kras* activation and evaluated the effect of *Raptor* deficiency in this model. We found that the addition of oncogenic *Kras* expression accelerated onset of Notch1-driven T-ALL. However, *Raptor* deficiency dramatically suppressed the propagation of leukemia cells even in this aggressive T-ALL model (Fig. S9D). These data demonstrate that complete mTORC1 inactivation, but not rapamycin treatment, is highly effective for the eradication of T-ALL cells in vivo.

Discussion

Essential Role of mTORC1 in Early T-Cell Progenitor Development.

Although rapamycin is a well-known allosteric inhibitor of mTORC1 and acts as an immunosuppressant, it has remained unclear how rapamycin affects T-cell development. Several studies showed that rapamycin blocks IL-2-dependent T-cell proliferation (25, 26). Because DN2 cells, but not DN1 cells, express an IL-2 receptor (IL-2R α ; CD25), mTORC1 was suggested to be activated at DN2. However, in this study, we found that both DN1 and DN2 showed increases in S6 phosphorylation and that mTORC1 deficiency by *Raptor* deletion caused a dramatic abnormality of cell cycling of DN1 cells, leading to developmental failure of DN2 and DN3. In contrast, although rapamycin induced remarkable thymic atrophy, similar to *Raptor* deficiency, it blocked differentiation from DN3 to DP, but not the DN1–DN2 transition; its effect on T-cell development thus appears to be different from the effect of loss of mTORC1 activation.

Prolonged treatment with rapamycin inhibits not only mTORC1, but also mTORC2 in some cell lines (27). In our experiment, although *Rictor* deficiency did not disrupt the development of early T-cell progenitors, DP cells failed to develop, indicating that mTORC2 is essential for T-cell development at later stages than mTORC1. Because we did not detect any reduction of 4E-BP1 phosphorylation in *Rictor*-deficient cells in our experimental setting, the defective phenotype of T-cell development caused by *Rictor* deficiency is independent of mTORC1. These data clearly demonstrate that mTORC1 and mTORC2 have distinct roles in T-cell development and suggest that the effects of rapamycin on T-cell development may be due to partial inhibition of mTORC1 activity, inhibition of mTORC2, or their combination.

Although a slight reduction of Mcl-1 protein was observed in normal T-cell progenitors and T-ALL cells after the *Raptor* deletion (Fig. S6A and Fig. S9B), we did not detect any sign of apoptosis (Fig. 2B; Fig. S6A; Fig. S9B). Mcl-1 is reported to be a potent downstream effector of mTORC1 signaling (28), but reduction of only this molecule may be insufficient to induce apoptosis in *Raptor*-deficient T-cell progenitors and T-ALL cells. One of the main reasons for the defective propagation of early T-cell progenitors with mTORC1 deficiency was the inhibition of the G1/S transition in DN1 cells. Evaluation of the expression of cell cycle regulators showed that Cyclin D2/D3-CDK6 protein levels were consistently reduced by *Raptor* deficiency in DN cells. Cyclin D3 is an essential D-type cyclin for normal expansion of T-cell progenitors (18). Cyclin D2 and D3 have nonredundant roles in T-cell development (29). Cyclin D3 deficiency is sufficient to reduce susceptibility to T-cell malignancies. Interestingly, Cyclin D3 deficiency reduces BM cellularity caused by defective expansion of granulocytes, but not of hematopoietic stem cells and GMPs (30), just as we observed in *Raptor*-deficient mice. Although T-ALL cells were efficiently eradicated when mTORC1 was inactivated in vivo, the reduction of CDK6 protein was not observed, suggesting that Cyclin D2 and D3 are major downstream targets of mTORC1. Some previous studies using rapamycin reported that the Cyclin D3 expression level is controlled by mTOR, but there were two possible mechanisms, i.e., translational regulation (31) and posttranslational regulation via the ubiquitin-proteasome pathway (32). In addition, 4E-BPs reportedly regulate mTORC1-mediated cell proliferation by translational control of Cyclin D3 in mouse embryonic fibroblasts (33). In our experiment, we found that mTORC1 deficiency, and not mTORC2 deficiency, induces protein degradation of Cyclin D2 and D3 mainly in a proteasome-dependent manner in T cells. These data suggest that Cyclin D2 and D3 are tightly regulated by mTORC1 activity in multiple ways. Deep understanding of the dependency of cell cycle regulation on the mTORC1-Cyclin D2/D3 pathway in different tissues will be important for successful mTORC1-targeted therapy.

Efficient Eradication of T-ALL, but Not Acute Myeloid Leukemia, by mTORC1 Inactivation. In contrast to the effective suppression of T-ALL progression by rapamycin, the cytostatic effect of rapamycin was not sufficient for the total eradication of T-ALL cells. The presence of rapamycin-insensitive mTORC1 substrates, like 4E-BP1, may account for this incomplete effect (13). Alternatively, the cytostatic effect of rapamycin on T-ALL may be mediated by mTORC2 inhibition because it was reported that *Rictor* deficiency significantly, but not completely, suppresses Notch-driven T-ALL (34), which is similar to the effect of rapamycin observed in our study. Higher doses of rapamycin *in vivo* may have stronger therapeutic efficacy for T-ALL, but may also have unexpected side effects because prolonged treatment with a higher dose of rapamycin inhibits mTORC2 as well as mTORC1 (12, 27, 35). Based on our findings, we believe that complete inhibition of mTORC1, but not mTORC2, would contribute to the therapeutic eradication of T-ALL.

In our previous study with an acute myeloid leukemia (AML) model, we showed that, although mTORC1 deficiency significantly suppresses leukemia progression by causing apoptosis of differentiated leukemia cells, mTORC1 does not control the cell cycle in undifferentiated AML cells *in vivo* (36). Therefore, we believe that a detailed comparative analysis of downstream target molecules between AML and T-ALL would lead to a deeper understanding of the molecular mechanisms by which mTORC1 controls the behavior of leukemia. Although several ATP-competitive mTOR inhibitors appear to have potent anticancer effects, a potential drawback to these agents is their inhibition of multiple

targets, which could lead to unwanted side effects or serious damage to normal tissues. Our study revealed that mTORC1-specific inhibition contributes to the eradication of T-ALL cells. The identification of targets downstream of mTORC1 or development of an mTORC1-specific inhibitor would contribute to the development of efficient and specific anticancer therapeutics.

Materials and Methods

Raptor^{fllox} mice were crossed with *Rosa26-CreER^{T2}* mice, *LSL-Kras^{G12D}* mice, *Lck-Cre* mice, and *Rosa26-tdRFP* reporter mice. *mTOR^{fllox}* mice, *Rictor^{fllox}* mice, and *Tsc1^{fllox}* mice were crossed with *Rosa26-CreER^{T2}* mice. Flow cytometric analyses were performed using monoclonal antibodies recognizing the appropriate cell-surface markers. Purified LSK cells from 8- to 12-wk-old mice were cultivated for 11–16 d with Tst-4DLL1 stromal cells (16). Cells expressing the *Rosa26-CreER^{T2}* gene were used as controls to exclude the effect of Cre activation. For the T-ALL model, cytokine-activated BM cells from mice were infected with a retrovirus carrying NICD-ires-GFP. Detailed descriptions of materials and methods can be found in *SI Materials and Methods*.

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