Mechanistically distinct cancer-associated mTOR activation clusters predict sensitivity to rapamycin

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Genomic studies have linked mTORC1 pathway–activating mutations with exceptional response to treatment with allosteric inhibitors of mTORC1 called rapalogs. Rapalogs are approved for selected cancer types, including kidney and breast cancers. Here, we used sequencing data from 22 human kidney cancer cases to identify the activating mechanisms conferred by mTOR mutations observed in human cancers and advance precision therapeutics. mTOR mutations that clustered in focal adhesion kinase targeting domain (FAT) and kinase domains enhanced mTORC1 kinase activity, decreased nutrient reliance, and increased cell size. We identified 3 distinct mechanisms of hyperactivation, including reduced binding to DEP domain–containing mTOR-interacting protein (DEPTOR), resistance to regulatory associated protein of mTOR–mediated (RAPTOR–mediated) suppression, and altered kinase kinetics. Of the 28 mTOR double mutants, activating mutations could be divided into 6 complementation groups, resulting in synergistic Rag- and Ras homolog enriched in brain–independent (RHEB–independent) mTORC1 activation. mTOR mutants were resistant to DNA damage–inducible transcript 1–mediated (REDD1–mediated) inhibition, confirming that activating mutations can bypass the negative feedback pathway formed between HIF1α and mTORC1 in the absence of von Hippel–Lindau (VHL) tumor suppressor expression. Moreover, VHL–deficient cells that expressed activating mTOR mutants grew tumors that were sensitive to rapamycin treatment. These data may explain the high incidence of mTOR mutations observed in clear cell kidney cancer, where VHL loss and HIF activation is pathognomonic. Our study provides mechanistic and therapeutic insights concerning mTOR mutations in human diseases.

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insufficient to cause ccRCC in various mouse models, indicating the requirement of additional genetic/epigenetic events (50–53). One candidate pathway is the mTOR signaling pathway. Evidence supporting such a scenario includes the following: (a) inhibitors of mTORC1 everolimus and temsirolimus are 2 standard treatment options for patients with metastatic RCC (mRCC), and (b) clustered, recurrent missense mutations of mTOR were observed in approximately 5% of cases of ccRCC (54–58). Hence, we envisioned that studying human kidney cancer–derived mTOR missense mutations could elucidate regulatory mechanisms of mTOR in cancers and other human diseases carrying mTOR mutations and affect treatment decisions.

Based on available human cancer genomic databases, there are approximately 570 missense mutations of mTOR identified across 20 cancer types that occur at various frequencies and at different amino acid positions (Supplemental Figure 1 and Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI86120DS1). These mutations present a challenge, but could also offer an opportunity to enroll cancer patients into histology-independent, genomics-guided, mutation-enriched “basket” trials (59). To implement this potential therapeutic strategy, an imperative step is to functionally interrogate cancer-derived mTOR missense mutations in a systemic fashion. Several regulatory mechanisms concerning the activation of WT mTORC1 have been proposed, which involves mTOR-interacting proteins RAG, Ras homolog enriched in brain (RHEB), DEP domain containing mTOR-interacting protein (DEPTOR), RAPTOR, PRAS40, and FKBP38 (2, 4–10, 19, 60–63). Indeed, a recent study surveyed cancer-derived mTOR activation mutations, which exploited the DEPTOR-centered mechanism (64). However, how activating mutations contribute to the pathogenesis of cancer, especially kidney cancer, and what molecular mechanisms underlie individual activating mutations beyond DEPTOR remain unknown.

Results
Kidney cancer–derived mTOR missense mutations clustered at FAT and kinase domains are activating mutations. The Cancer Genome Atlas Kidney Renal Clear Cell Carcinoma (TCGA-KIRC) project, consisting of stage I–IV cases (stages I–II, 257 cases [57.6%]; stages III–IV, 189 cases [42.4%]), has identified mTOR missense mutations at 5.4% (54). The RECORD-3 trial (Renal Cell Cancer Treatment with Oral RAD001 Everolimus Given Daily) (65), consisting of all stage IV cases (stage IV, 258 cases sequenced [100%]), identified mTOR missense mutations at 6% (P = 0.67, t test, comparison between the mutation rates between 2 studies) (Figure 1A and Supplemental Table 1) (65, 66). Together, these findings support
toward S6K than 4EBP based on phosphorylation was observed among mTOR-activating mutations, which might be due to differential "substrate quality" between S6K and 4EBP (67). These results suggest that S6K could discern differences of mTORC1 activity better than 4EBP. Hence, we mainly utilized phosphorylated S6K as the readout of mTORC1 activity for this study. Similar results on S6K and AKT phosphorylation were obtained when individual mTOR constructs were expressed at a level comparable to that of endogenous mTOR protein using an mTOR-silenced 293T cell line (Supplemental Figures 4 and 5). To determine the sensitivity of these hyperactive mTOR mutants to rapamycin, we generated 6 HeLa cell lines expressing tetracycline-inducible WT or mutant mTOR that corresponds to different mutation clusters (Supplemental Figure 6). Notably, all these hyperactive mTOR mutants remained sensitive to rapamycin (Figure 1D), which is consistent with prior reports (30, 31, 64).

mTOR-activating mutants are more resistant to glucose and serum but not to amino acid deprivation than WT mTOR. Diverse nutrient and growth factor signals converge on small GTPases RAG and RHEB to coordinate mTORC1 activation (2, 19). Amino acids activate RAGA/B GTP:C/DGDP, which recruits mTORC1 the idea that hyperactive mTORC1 resulting from mTOR-activating mutations could constitute an oncogenic driver event in kidney cancer pathology (53).

Although mTOR missense mutations scattered through the protein in most cancers, they clustered within the focal adhesion kinase targeting domain (FAT) and kinase domains in kidney cancer from these 2 independent studies (Figure 1, A and B). To investigate the functional consequence, we generated 22 individual missense mTOR mutants and examined their impact on mTORC1 activity by assessing the phosphorylation of S6K and 4EBP1. When individual mTOR mutants were coexpressed with HA-S6K or HA-4EBP in 293T cells, the majority of FAT and kinase domain mutants exhibited higher activity, whereas HEAT domain mutants exhibited similar mTORC1 kinase activity with respect to WT mTOR (Figure 1C and Supplemental Figure 2). Meanwhile, when individual mutants were coexpressed with HA-AKT1, most mutants exhibited activity similar to that of WT mTOR (Supplemental Figure 3). Therefore, in this study we denote mTOR mutations exhibiting significantly higher mTORC1 activity than WT mTOR as activating mutations and those with similar activity as nonactivating mutations. Notably, a wider range of activities toward S6K than 4EBP based on phosphorylation was observed among mTOR-activating mutations, which might be due to differential "substrate quality" between S6K and 4EBP (67). These results suggest that S6K could discern differences of mTORC1 activity better than 4EBP. Hence, we mainly utilized phosphorylated S6K as the readout of mTORC1 activity for this study. Similar results on S6K and AKT phosphorylation were obtained when individual mTOR constructs were expressed at a level comparable to that of endogenous mTOR protein using an mTOR-silenced 293T cell line (Supplemental Figures 4 and 5). To determine the sensitivity of these hyperactive mTOR mutants to rapamycin, we generated 6 HeLa cell lines expressing tetracycline-inducible WT or mutant mTOR that corresponds to different mutation clusters (Supplemental Figure 6). Notably, all these hyperactive mTOR mutants remained sensitive to rapamycin (Figure 1D), which is consistent with prior reports (30, 31, 64).

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comparable in size under full media, mTOR-mutant HeLa cells were larger than mTOR-WT HeLa cells upon glucose or serum deprivation (Figure 2D). Consistent with the complete dependence of mutant mTORC1 activity on amino acid as WT mTORC1 (Figure 2C and Supplemental Figure 7C), mTOR mutant HeLa cells were comparable in size to mTOR-WT HeLa cells upon amino acid deprivation (Figure 2D). Collectively, our data demonstrated that kidney cancer–derived mTOR-activating mutations appear to sustain mTORC1 kinase activity under glucose- or serum-limited but not amino acid–limited conditions.

All cancer-derived mTOR-activating mutants require RAG and RHEB for activation. The crystal structure of mTOR was recently reported, which reveals that the FAT domain forms a C-shaped solenoid that wraps around and clamps the V-shaped kinase domain (Figure 3A) (80). This level of structural resolution enabled us to accurately position individual mTOR mutations and allow for structure-based assignment of 6 distinct clusters, including 3 in the FAT domain (F1, F2, F3) and the other 3 in the kinase domain (K1, K2, K3) (Figure 3A). Several regulatory mechanisms concerning the activation of WT mTORC1 have been proposed, including mTOR-interacting proteins RAG, RHEB, DEPTOR, RAPTOR, PRAS40, and FKBP38 (2, 4–10, 19, 60–63) (Figure 1B). We hypothesized that distinct mTOR mutation clusters might affect the interaction/regulation between mTOR and specific mTOR regulators, contributing to mTORC1 activation.

The activation of mTORC1 requires RAG and RHEB, which mediate lysosome localization and direct activation, respectively (2, 76). Accordingly, we tested to determine whether overexpression of dominant negative RAGB GDP/RAGD GTP (61) or knockdown of RHEB affected the activity of mTOR mutants in phosphorylating S6K. All the activating mTOR mutants were sensitive to RAGB GDP/RAGD GTP (Figure 3B), which is consistent with the fact that these mTOR mutants were sensitive to amino acid deprivation (Figure 2D). Collectively, our data demonstrated that kidney cancer–derived mTOR-activating mutations appear to sustain mTORC1 kinase activity under glucose- or serum-limited but not amino acid–limited conditions.

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All mTOR-activating mutants are sensitive to negative regulator PRAS40, yet display differential binding to negative regulator DEPTOR. We next examined the effect of 3 reported negative regulators of mTORC1, PRAS40, FKBP38, and DEPTOR, on activating mTOR mutants. Overexpression of PRAS40 inhibited all the tested mTOR mutants from phosphorylating S6K (Figure 4A), which is consistent with the known mechanism of PRAS40 that occurs through disrupting RAPTOR-mediated mTORC1 substrate recognition, and the necessity of RAPTOR in mTORC1 signaling (5, 6, 10). Neither overexpression nor knockdown of FKBP38 affected the activity of WT and mTOR mutants (Figure 4, B and C). Of note, the role of FKBP38 as a negative regulator of mTORC1 remains highly debated (81, 82). DEPTOR is reported to bind to the FAT domain of mTOR and negatively regulate mTORC1 (4). Coinmunoprecipitation assays were performed to assess the interaction between individual mTOR mutants and DEPTOR. Notably, mTOR mutants in the F1 (L1460P, C1483F) but not F2 or F3 cluster coprecipitated less DEPTOR than WT mTOR (Figure 4D). As DEPTOR overexpression has been shown to inhibit WT mTORC1 activity (4), we examined the ability of DEPTOR overexpression to inhibit individual mTOR mutants. We showed that mTORC1 activities of L1460P (F1) and C1483F (F1) mutants were reduced when DEPTOR was overexpressed (Supplemental Figure 8), which suggests that DEPTOR may directly bind and thereby inhibit mTOR near the F1 cluster. Reduced binding to DEPTOR was also observed in 1 mTOR mutant in the K3 cluster (R2505P) (Figure 4D), which is consistent with other reports (64, 83). However, overexpression of DEPTOR did not inhibit the activity of the mTOR R2505P mutant (Supplemental Figure 8), implicating an indirect mechanism.

Subsets of mTOR-activating mutants are resistant to RAPTOR-mediated suppression. RAPTOR defines mTORC1 and presents substrates to mTOR, yet paradoxically negatively regulates mTORC1 upon overexpression or under low nutrient conditions (6). Accordingly, we examined the ability of RAPTOR overexpression to inhibit individual mTOR mutants, which led to the discovery of RAPTOR-sensitive and -insensitive mutants (Figure 5A). Similarly to WT mTOR, the mTORC1 activity of F1888L (F2), L2230V (K2), M2327I (K3), and R2505P (K3) mutants was reduced when RAPTOR was overexpressed. In contrast, the activity of L1460P (F1), C1483F (F1), T1977K (F3), and S2215F (K1) was not
affected by RAPTOR overexpression. These data suggest that amino acid residues in the F1, F3, and K1 clusters of mTOR might be important for sensing the negative inhibition exerted by RAPTOR. Interestingly, a recently solved mTORC1 structure consisting of mTOR and RAPTOR supports the interaction topology between mTOR and RAPTOR and features the roles of RAPTOR not only in substrate presentation but also in active site restriction (84).

**mTOR kinase domain mutants exhibit increased kinase activity and display structural reorganization.** Conceivably, the mutations in kinase domain may alter the conformation to affect the enzyme kinetics. Hence, we determined the enzyme kinetics of S2215F (K1) and L2230V (K2) mTOR mutants in phosphorylating S6K (Figure 5, B and C). Interestingly, S2215F increased \( V_{\text{max}} \) and decreased \( K_m \) of mTORC1 kinase activity, whereas L2230V only affected \( V_{\text{max}} \) (Table 1). Since the crystal structure of C-terminal mTOR has been determined (80), we sought to examine the possible effects of the mTOR mutations on the conformation of C-terminal mTOR. Specifically, we performed 10 replicate molecular dynamics simulations for S2215F mutant; each trajectory was approximately 500 nanoseconds, and subsequent simulation data were analyzed for structural alternations indicative of rapid mutation-induced conformational changes. In a number of these simulations, we observed a displace-

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**Figure 5. Characterization of the response of mTOR-activating mutants to RAPTOR-mediated inhibition; mTOR kinase domain mutants exhibit increased kinase activity and display structural reorganization.**

(A) 293T cells, transfected with vectors expressing HA-S6K, indicated Flag-mTOR mutants, and either GFP or RAPTOR was subjected to immunoblot analysis using the indicated antibodies. Hatched bars indicate the mutants not inhibited by overexpressed RAPTOR. Densitometry of phosphorylated S6K versus HA-S6K from 3 independent experiments is shown (mean ± SEM, \( n = 3 \) independent experiments). **\( P < 0.01 \); ***\( P < 0.001 \) (t test). (B) In vitro kinase assays were carried out with the indicated Flag-tagged recombinant mTOR proteins in the presence of the indicated amount of recombinant HA-S6K. The recombinant Flag-mTOR and HA-S6K proteins were produced in 293T cells by transient transfection of respective expression constructs followed by anti-Flag and anti-HA affinity purification, respectively. The reactions were analyzed by the indicated immunoblots. (C) Graph shows the quantitation of the phosphorylated S6K(T389) determined by densitometry versus S6K at different concentrations. (D) A contact map where each point \((x, y)\) gives the net change in probability of forming a contact between residue \(x\) and residue \(y\) between the mutant and WT, as estimated from simulations. The net contact change (blue indicating gain, red indicating loss) observed in the S2215F mutant is shown in the upper-left triangle, with WT shown in the bottom right to indicate the secondary structure present at corresponding locations. Disrupted contacts between residues 2214 and 2217 (green circle 1), residues 2218 and 2389, and residues 2214 and 2402 (green circle 2) are highlighted. (E) Superposition of WT (gray) and S2215F (wheat) mTOR kinase domain structures at 501 ns of simulation, with the S2215F mutation shown in red sticks. Of note, ku3b is unwound and has moved further away from ku8 in S2215F simulation.
Kidney cancer–derived mTOR-activating mutants are resistant to REDD1-mediated inhibition and promote rapamycin-sensitive tumor formation in vivo. Clear cell kidney cancer is genetically noted for the biallelic inactivation of VHL tumor suppressor, which leads to the stabilization and accumulation of HIFs (37, 50, 54). DNA damage–inducible transcript 1 (REDD1) is a key transcriptional target of HIF1, and it negatively regulates mTORC1 (Supplemental Figure 10) (86–88). REDD1 activates TSC1/2 to inactivate RHEB and thereby inhibits mTORC1. On the other hand, active mTORC1 increases HIF1 protein levels through enhanced translation (89), which in turn induces REDD1, serving as a negative feedback loop (90). Based on this intricate interplay between HIF1 and mTORC1, we envisioned that mTOR-activating mutations might oppose REDD1-mediated inhibition and thereby abrogate the negative feedback loop linking HIF and mTOR. Indeed, overexpression of REDD1 was able to inhibit the WT mTOR, but not any of the tested activating mTOR mutants except F1888L (Figure 6A). The ability of activating mTOR mutations to escape negative regulation by REDD1 helps explain why mTOR-activating mutations occur frequently in clear cell kidney cancer in which REDD1 is upregulated by HIF in lieu of the VHL loss.

We next investigated whether these mTOR-activating mutations render tumor growth advantages in vivo. To assess potential phenotypic collaboration between VHL loss and mTORC1 hyperactivity, we depleted VHL using shRNA and overexpressed either WT or activating mutant mTOR in NIH/3T3 cells. As indicated by higher phosphorylated S6K, VHL-depleted NIH/3T3 cells expressing activating mTOR mutants displayed higher mTORC1 activity than those expressing WT mTOR (Figure 6B and Supplemental Figure 11). Furthermore, tumor allografts demonstrated that VHL-depleted NIH/3T3 cells expressing activating mTOR mutants (C1483F, S2215F, F1888L, T1977K, L2230V, and R2505P), but not nonactivating mutant (A1105P), grew significantly faster than those expressing WT mTOR (Figure 6, C and D and Supplemental Figures 12 and 13). To our knowledge, this is the first in vivo evidence demonstrating that mTOR-activating mutants directly contribute to tumor growth. Importantly, these tumor allografts were sensitive to rapamycin treatment (Figure 6, E and F).

It was reported that REDD1 activates TSC1/2 by sequestering 14-3-3 and thereby frees TSC1/2 to inactivate RHEB at the lysosome despite the inhibitory phosphorylation of TSC2 by upstream kinases at Ser939 (74, 88). Accordingly, we examined the TSC2 phosphorylation in VHL-depleted NIH/3T3 cells expressing WT or activating mutant mTOR (Supplemental Figure 14). Ser939 of TSC2 was strongly phosphorylated in VHL-depleted cells expressing either WT or mutant mTOR. Furthermore, overexpression of REDD1 was unable to suppress the tumor growth of VHL-depleted NIH/3T3 cells expressing activating mTOR C1483F mutant (Supplemental Figure 15). These results further support the notion that mTOR-activating mutations function downstream, bypass REDD1-TSC2–mediated inhibition, and thereby abrogate the negative feedback loop linking HIF and mTOR.

mTOR-activating mutants from different clusters display functional synergism. Collectively, we demonstrated 3 distinct mechanisms employed by specific mTOR mutation clusters to activate mTORC1, i.e., the loss of DEPTOR-dependent inhibition, the escape from RAPTOR suppression, and the increase of intrinsic kinase activity. All of these mechanisms could contribute to aberrant mTORC1 activity and thereby promote tumorigenesis. We hypothesized that functional complementation might occur between mechanistically distinct clusters, which might reveal not only the interdependence of individual activation mechanisms, but also the uncharacterized, potential mechanisms. To test this hypothesis, we systemically generated 28 mTOR mutants that carry 2 mutations from either the same or different clusters within a single mTOR molecule and determined their activity based on S6K phosphorylation (Figure 7, A and B). Remarkably, most double mutations across different clusters synergized strongly except for those involving the K3 cluster (Figure 7, A–C). Importantly, double mutations within the same clusters F1 (L1460P and C1483F) and K3 (M2327I and R2505P) did not yield synergism (Figure 7, A and B). Interestingly, F1888L (F2), a FAT domain mutation with no discernible features with respect to DEPTOR interaction and RAPTOR inhibition, synergized with all other mutations, thereby implicating yet-uncharacterized activating mechanisms. Overall, these data suggest that the geographically assigned mutation clusters coincide with the functional complementation groups.

mTOR mutants with 2 mutations from distinct clusters are hyperactive without RAG and RHEB. The strong synergism observed in mTOR double mutants prompted us to investigate their reliance on RAG and RHEB, their resistance to PRAS40, and their sensitivity to nutrient deprivation. Surprisingly, the examined mTOR double mutants were resistant to the overexpression of dominant negative RAGB<sup>GDP</sup>/RAGD<sup>GTP</sup> (Figure 8A and Supplemental Figure 16) and to the knockdown of RHEB (Figure 8B and Supplemental Figure 17), suggesting the loss of reliance on both RAG and RHEB for mTORC1 activation. On the other hand, these mutants were still inhibited by PRAS40 overexpression (Figure 8C and Supplemental Figure 18), indicating that synergistic double mutations did not alter substrate recognition through the TOR signaling (TOS) motif (91). Moreover, increased resistance of mTORC1 signaling to glucose, serum, or amino acid deprivation was observed in mTOR (C1483F/T1977K, C1483F/S2215F, T1977K/S2215F, and F1888L/L2230V) mutant HeLa cells (Figure 9, A–C and Supplemental Table 1. Kinetic measurement of mTORC1 (WT or mutants) in vitro kinase activity toward S6K

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<th>WT</th>
<th>S2215F</th>
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<td>$V_{\text{max}}$ (AU)</td>
<td>9.5 ± 0.4</td>
<td>15.5 ± 1.6</td>
<td>16.8 ± 1.7</td>
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<td>$K_{\text{m}}$ (μM)</td>
<td>5.1 ± 0.6</td>
<td>0.9 ± 0.4</td>
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eukaryotic cells to switch between anabolism and catabolism (2, 25, 92). Accurate mTOR signaling is important for normal cellular homeostasis in metabolism, and dysregulation can result in diverse human diseases, including cancer, obesity, diabetes, and neurological disorders, as well as aging (1, 2, 93–95). Unlike mutations of the other PI3K/AKT/mTOR pathway components that have been extensively studied, the presence, the functional outcome, and the underlying activating mechanisms of mTOR missense mutations in contributing to human illness remain to be investigated. Recent mTORC1 inhibitor outlier studies in ccRCC, urothelial/bladder cancer, and thyroid cancer demonstrated that.

**Discussion**

As a central player in the PI3K/AKT/mTOR pathway, mTOR functions as an integrator of intracellular and extracellular signals in
HEAT domains are nonactivating mutations, which is consistent with previous reports (64, 83, 96, 97). Of note, mutations from different clusters could show different activities toward S6K in vitro, which is also reflected by the different tumor growth rates in vivo. These results further support that mutants of different clusters can be activated by different mechanisms. On the other hand, we found these mutations have little effect on mTORC2 activities. One study suggested mTOR mutations could increase mTORC2 activities, but the effects were very subtle (83), which is largely in line with our findings. Importantly, all activating mutations are sensitive to rapamycin treatment. Hence, cancer patients could be selected for mTOR inhibitor treatment based on mTOR mutations for basket trials (59). Interestingly, mTOR-activating mutations have recently been reported to associate with focal cortical dysplasia type II, which can lead to intractable epilepsy (98) that can be pharmacologically managed with rapamycin. This example and many yet to be discovered highlight roles of mTOR-activating mutations in human diseases beyond cancer and underscore the importance in characterizing and elucidating functional outcomes and the respective activating mechanisms of individual mTOR missense mutations.

Direct mTORC1 activation through either complete TSC1 loss or mTOR-activating mutation was associated with long-term therapeutic benefit (30–33). Furthermore, one recent study reported that mutations in mTOR, TSC1, or TSC2 were more common in responders than nonresponders; however, a substantial fraction of responders had no mutations in the mTOR pathway (34).

Through systemic characterization of human kidney cancer–derived mTOR mutations, we demonstrated that mutations clustered at FAT and kinase domains are activating mutations for upregulating mTORC1 signals, while those dispersed at the sites of mTOR-activating mutations in human diseases beyond cancer and underscore the importance in characterizing and elucidating functional outcomes and the respective activating mechanisms of individual mTOR missense mutations.

Upstream signaling relays contributing to the eventual mTORC1 signaling output are complex (1, 19, 99). Nevertheless, they mainly converge on 2 regulatory complexes of mTORC1, i.e. the Ragulator/RAG axis for amino acids and the TSC1/2/RHEB axis for growth factor, receptor signaling, and other stress signals (1, 19). Interestingly, all of the mTOR-activating mutations
responder whose tumors carried mTOR-activating mutations (30, 31). Furthermore, the important collaboration between VHL loss (HIF activation) and mTORC1 activation presented by the current study in kidney cancer biology helps explain why kidney cancer was the first FDA-approved cancer type for rapalog-based therapies. Admittedly, it should be noted that activating of mTOR may be achieved through various means not limited to activating mutations in mTOR and the tumor heterogeneity may also complicate the usage of mTOR-activating mutations as biomarkers for predicting rapalog response (53). Nevertheless, our data indicate that when mTOR-activating mutation is an early “driver event” for tumorigenesis, it predicts the rapalogs response very well (101). Some mTOR-activating mutations characterized in this study were also detected in other cancer types (for example, S2215F was also identified in cervical squamous cell carcinoma, colorectal adenocarcinoma, and melanoma), and the role of these mTOR-activating mutations in the pathogenesis of other cancer types remains to be determined. However, as hypoxia is a common feature of most tumors (102, 103), mTORC1 activation may help tumor cells overcome the physiological brake on anabolism initiated by tissue hypoxia. A recent study also demonstrat-

Figure 8. Characterization of the dependency of mTOR double mutants on RAG and RHEB. (A) 293T cells were transfected with vectors expressing HA-S6K, the indicated Flag-mTOR mutants, and either RAGB plus RAGD (RAGB/DWT) or dominant negative RAGB<sup>CP</sup> plus RAGD<sup>CTP</sup> (RAGB/DMut). Whole cell lysates were analyzed by the indicated immunoblots. (B) HeLa cells stably expressing shRNA against GFP or RHEB were transfected with vectors expressing HA-S6K and the indicated Flag-mTOR mutants and analyzed by the indicated immunoblots. (C) 293T cells were transfected with vectors expressing HA-S6K, the indicated Flag-mTOR mutants, and either GFP or PRAS40 and analyzed by the indicated immunoblots. For A–C, densitometry of phosphorylated S6K versus HA-S6K from 3 independent experiments is shown (mean ± SEM, n = 3 independent experiments). *P < 0.05; **P < 0.01; ***P < 0.001 (t test).
our data, cluster F1 may define the region of mTOR interacting with DEPTOR and mutations (L1460P, C1483F) in this region could affect DEPTOR’s direct binding to mitigate its negative regulation. Meanwhile, mutations of cluster F1 that map to TRD1 and the TRD1-proximal portion of TRD2 might destabilize interaction between TRD1 and the kinase domain. Cluster F3 maps to the HRD domain, and mutations of this cluster (T1977K) might also destabilize interaction between HRD and the kinase domain. Presumably, these kinds of destabilizations could lead to more openings for substrates to access the catalytic core. Mutations in the kinase domain (S2215F, L2230V) could increase the intrinsic catalytic rate ($V_{\text{max}}$), and the others (S2215F) could increase the substrate access. These changes are likely to be caused by the conformational rearrangement induced by individual mutations. In another case, arginine-to-proline change of the mutation R2505P (cluster K3) could disrupt the α-helix structure and distort the normal function of the negative regulatory loop nearby (104). Overall, all these results support the idea that individual mTOR mutation clusters can be activated by different mechanisms. Altogether, we exploited an approach based on the genetic complementation assay to examine the interplay among individual activating mecha-

Figure 9. Characterization of nutrient dependence and sensitivity to rapamycin of mTOR double mutations. (A–C) HeLa cells expressing tetracycline-inducible WT or mutant mTOR were treated with doxycycline for 48 hours to induce mTOR. Cells were then starved for glucose (A), serum (B), or amino acids (C) for 1 hour or starved for 1 hour and restimulated with full media for 1 hour and subjected to immunoblot analysis. Densitometry of phosphorylated S6K versus HA-S6K from 3 independent experiments is shown (mean ± SEM, $n = 3$ independent experiments). *P < 0.05; **P < 0.01; ***P < 0.001 (t test). *P < 0.05; **P < 0.01; ***P < 0.001 (t test, comparison between indicated mutants and WT). (D) Tetracycline-inducible HeLa cells expressing WT or mutant mTOR under the indicated culture conditions were subjected to flow cytometry analysis for cell size. Data shown are the mean FSC-H from 3 independent experiments. Error bars represent SEM. *P < 0.05; **P < 0.01 (t test). (E) Tetracycline-inducible HeLa cells expressing WT or mutant mTOR were treated with the indicated doses of rapamycin for 1 hour prior to immunoblot analysis using the indicated antibodies.
anisms. Our results demonstrated that, in principle, 2 mutations from different mechanistic clusters cooperated, while 2 from the same did not. Furthermore, additional regulatory mechanisms beyond what we have presented might exist and warrant future investigation. Of note, mTOR belongs to the PI3K-related kinase (PIKKs) family, which has sequences similar to PI3K (105) and therefore may share the similar regulatory mechanisms with each other. Indeed, mutations in helical and kinase domains of the p110 subunit are activated by different mechanisms and can synergize when present in the same p110 molecule (106).

Intriguingly, cooperative double mTOR mutants became RAG and RHEB independent in mTORC1 activation. This is the only report, to our knowledge, showing this possibility. RHEB is the direct activator of mTORC1, but how this small GTPase activates mTOR remains elusive. Our study of RHEB-independent mTOR double mutants supports a potential mechanism by which RHEB activates mTORC1, i.e., RHEB functions to gauge upstream signaling strength and thereby gradually releases various restricted measures for adequate mTORC1 signaling output. Interestingly, mTOR double mutants display elevated resistance to low-dose rapamycin (2.5 nM), but remain sensitive to high-dose rapamycin (5–25 nM). It is tempting to speculate that, as limited drug perfusion of solid tumor could lead to subtherapeutic concentrations of rapalogs in poorly perfused tumor areas (107), tumor cells with single mTOR mutations could develop resistance to rapalog treatment by acquiring additional, mechanistically distinct mTOR mutations, which warrants further investigations. Our study and others on cancer-derived mTOR-activating mutations has not only deciphered a mechanistic blueprint concerning how mTORC1 is regulated, but also has laid the mechanistic basis for selecting patients whose diseases carry mTOR-activating mutations for the treatment of mTOR inhibitors.

Methods

Plasmid construction and shRNA- and siRNA-mediated knockdown. See Supplemental Methods for detailed information on plasmids, shRNA constructs, and siRNAs.

mTOR single mutations or double mutations were generated by introducing corresponding nucleotide changes into pcDNA3-Flag-mTOR using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent). See Supplemental Table 3 for primers used for site-directed mutagenesis. All the constructs were confirmed by DNA sequencing. Lentiviral vectors carrying the indicated shRNA were cotransfected with pCMV8.BR and pHCMV.VSVG into 293T cells to generate lentivirus. Cells infected with lentivirus were under puromycin selection at 10 μg/ml and lysed 1 hour later as described above.

Cell culture, transfection, and immunoblot analysis. 293T (ATCC), HeLa (ATCC), and NIH/3T3 (ATCC) cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS, nonessential amino acids, L-glutamine, sodium pyruvate, and antibiotics (Invitrogen). When indicated, cells were cotransfected with vectors expressing RAPTOR (3 μg), DEPTOR (3 μg), PRAS40 (1 μg), RAGB (200 ng), RAGD (200 ng), RAGBΔGR (200 ng), RAGDΔGR (200 ng), or FKBP38 (1 μg). At 48 hours after transfection, cells were harvested in ice-cold PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4), pelleted, and lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 1% Na deoxycholate, 0.01 M sodium phosphate, pH 7.2, 0.1% SDS, 2 mM EDTA, 50 mM NaF) containing complete protease inhibitor (Roche) and phosphatase inhibitors (EMD/Millipore). Protein concentration was determined by the BCA Kit (Pierce). Equal amounts of proteins (20–40 μg) were resolved by 10% or 4%–12% NuPAGE (Life Technologies) and transferred onto PVDF membrane (Immobilon-P, Millipore). Antibody detection was accomplished using the enhanced chemiluminescence method (Western Lightning, PerkinElmer) and the LAS-3000 Imaging System (Fujifilm). The immunoblot data were analyzed using ImageGauge software (Fujifilm).

See Supplemental Methods for detailed information on antibodies.

Generation of tetracycline-inducible HeLa and NIH/3T3 cell lines expressing WT or mutant mTOR. See Supplemental Methods for detailed information.

Nutrient deprivation and rapamycin treatment. For nutrient deprivation and refedding, tet-inducible HeLa cell lines expressing WT or mutant mTOR were treated with doxycycline. Forty-eight hours later, cells were rinsed briefly with PBS, treated with complete DMEM media devoid of serum (for serum starvation), glucose (for glucose deprivation), or amino acid (for amino acid deprivation) for 1 hour, and restimulated with full complete media for 1 hour as indicated. Subsequently, cells were harvested and lysed as described above.

Rapamycin was obtained from Sigma-Aldrich (catalog R8781). Tet-inducible HeLa cell lines expressing WT or mutant mTOR were treated with the indicated concentrations of rapamycin or vehicle (DMSO) 48 hours after induction with doxycycline. Cells were harvested and lysed 1 hour later as described above.

Coimmunoprecipitation and in vitro kinase assays. See Supplemental Methods for detailed information.

Cell size measurement by flow cytometry. Cells were treated as indicated, harvested, and analyzed on a flow cytometer using the parameter mean forward scatter height (FSC-H), which is a measure of relative cell size. Flow cytometry was performed using a LSRFortessa (BD Biosciences), and data were analyzed using FACSDiva (BD Biosciences).

Molecular dynamics simulations. See Supplemental Methods for detailed information.

Mouse allograft study. For subcutaneous growth, 5 million NIH/3T3 cells expressing shRNA constructs and the tet-inducible mTOR were mixed 2:1 with Matrigel (BD Biosciences) and injected subcutaneously into 2 flanks of 6- to 8-week-old female NOD-SCID IL-2Rγ (NSG) mice (The Jackson Laboratory, stock no. 005557). Animals were administered with doxycycline (2 mg/ml; Sigma-Aldrich) plus sucrose (50 mg/ml) in their drinking water to induce mTOR. The dimension of the tumors was measured by calipers, and tumor volume was calculated by the following formula: ½ × width2 × length.

For drug treatment, 5 million NIH/3T3 cells expressing shRNA constructs and the tet-inducible mTOR were mixed 2:1 with Matrigel (BD Biosciences) and injected subcutaneously into 2 flanks of 6- to 8-week-old male NSG mice. When tumor size reached 100 mm3, mice were randomized into 2 groups and were treated with either rapamy-
cin (8 mg/kg) or vehicle (0.25% PEG-400, 0.25% Tween-80) 3 days per week by intraperitoneal injection as previously reported (108).

Statistics. Results are presented as mean ± SEM. Except where otherwise specified, statistical significance was determined by unpaired 2-tailed Student’s t test. Except where otherwise specified, a P value of less than 0.05 was considered significant.

Study approval. All mouse experiments were performed using a protocol approved by the IACUC at Memorial Sloan Kettering Cancer Center (protocol no. 10-07-012). All animal work was performed in accordance with NIH guidelines (Guide for the Care and Use of Laboratory Animals. National Academies Press. 2011) and in compliance with all Memorial Sloan Kettering Cancer Center institutional requirements.

Author contributions
Design research studies were performed by JX, ZY, NR, EHC, and JJH. Conduct experiments were performed by JX, CGP, SKA, YD, TO, CHL, and DLP. Data acquisition was performed by JX, SKA, and DC. Data analysis was performed by JX, SKA, JDC, ZY, NR, EHC, and JJH. The manuscript was written by JX, EHC, and JJH. JX, ZY, SH, VRO, and JJH provided reagents. Study supervision was by JJH.

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3. Kim DH, et al. GbetaL, a positive regulator of the SH, VRO, and JJH provided reagents. Study supervision was by JJH.


