SUPPLEMENTARY FIGURE LEGENDS

Figure S1: Commercially available mTOR P-S2481 antibodies are site- and phospho-specific. Site-specificity (left panel): HEK293 cells were transfected with vector control (V), wild-type (WT) [1.0 μg], or S2481A [1.0 μg] Myc-mTOR alleles, as indicated. WCL was immunoprecipitated with Myc antibodies and immunoblotted with the indicated antibodies. Phospho-specificity (right panel): HEK293 WCL was immunoprecipitated with anti-mTOR antibodies (lanes 1-3). The immunoprecipitates were resuspended immediately in sample buffer (lane 1) or washed in phosphatase buffer and incubated in the presence (lane 2) or absence (lane 3) of λ-phosphatase [250 units] for 30 min. The immunoprecipitates were immunoblotted as indicated.

Figure S2: mTOR S2481 autophosphorylation is not required for mTORC1-mediated phosphorylation of S6K1 *in vivo*. HEK293 cells were transiently transfected with vector control (lane 1) or co-transfected with HA-S6K1 [0.5 μg] plus various Myc-mTOR alleles [5 μg], as indicated (lanes 2-12) (Myc-mTOR-WT, -RR, -RR/S2481A, or -RR/KD). Transfected cells were serum deprived for ~20 hr, pre-treated without or with rapamycin [20 ng/mL] for 30 min, stimulated with insulin for 30 min, and lysed in Buffer A with NP40-Brij35. WCL was immunoprecipitated with HA antibodies and immunoblotted as indicated. WCL was also immunoblotted directly to confirm equivalent expression of Myc-mTOR alleles. Note abbreviations: WT: wild type; RR: rapamycin-resistant; KD: kinase dead.

Figure S3: mTOR S2481 autophosphorylation is not required for mTORC1-induced dissociation of 4EBP1 from eIF4E, a read-out of mTORC1-dependent 4EBP1 phosphorylation. mTORC1 signaling mediates multi-site phosphorylation of 4EBP1 (e.g. on several sites, T37/46, S65, T70), a translational repressor that binds and inhibits the translation initiation factor eIF4E (1). eIF4E constitutively binds the cap structure at the 5' end of mRNAs to initiate cap-dependent translation in cooperation with other initiation factors in the 4EBP1-free state. Thus, insulin induces mTORC1-mediated dissociation of 4EBP1 from eIF4E, which occurs in a rapamycin-sensitive manner. m^7GTP mimics the 5' cap structure, and when coupled to sepharose beads, functions as an eIF4E affinity matrix. In this experiment, HEK293 cells were transiently transfected with vector control (lane 1) or co-transfected with HA-4EBP1 [0.5 μg] together with various AU1-mTOR alleles [5 μg], as indicated (lanes 2-15) (AU1-mTOR-WT, -RR, -RR/KD, or -RR/S2481A). Transfected cells were serum deprived for ~20 hr, pre-treated without or with rapamycin [20 ng/mL] for 30 min, stimulated with insulin for 30 min, and lysed in Buffer A with NP40-Brij35. WCL was incubated with m^7GTP sepharose beads to affinity-purify ("pull-down") eIF4E and associated 4EBP1. The affinity-purified material was then immunoblotted as indicated. WCL was also immunoblotted directly to confirm equivalent expression of the transfected proteins. As in Figure S2, the combination of rapamycin and RR-mTORs allowed the examination of *in vivo* signaling by exogenously expressed, phosphorylation site-defective AU1-mTOR-S2481A in the absence of signaling by endogenous mTORC1.

Figure S4: S2481 autophosphorylation does not modulate the interaction of mTOR with raptor or mLST8. HEK293 cells were co-transfected with Myc-mTOR [4 μg], HA-raptor [0.5 μg], and HA-mLST8 [0.5 μg], serum deprived, and stimulated with insulin, as indicated. WCL was immunoprecipitated with Myc antibodies and immunoblotted as indicated (upper panels). WCL was also immunoblotted directly to confirm the expression of the various exogenously expressed proteins (lower panels).

Figure S5: Kinetics of insulin-stimulated, mTORC1-associated mTOR P-S2481. HEK293 cells were serum deprived and stimulated with insulin for various amounts of time, 5-120 min (left panel) or 2-15 min (right panel). WCL was immunoprecipitated with anti-raptor antibodies and immunoblotted with the indicated antibodies. WCL was also immunoblotted directly (lower panels) with the indicated antibodies

to confirm the expected activation of mTORC1 signaling. Note: The left and right panels represent independent experiments.

SUPPLEMENTARY REFERENCES

1. Ma, X. M., and Blenis, J. (2009) *Nat Rev Mol Cell Biol* 10(5), 307-318

Soliman_Supplemental Figures S1-S5

Figure S1

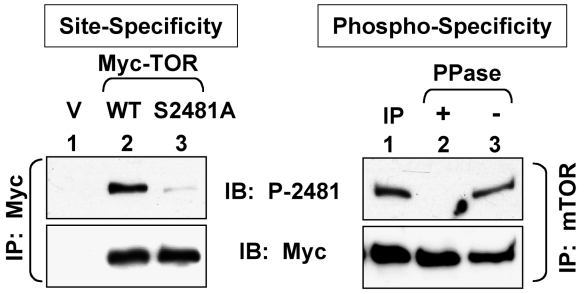

Figure S2

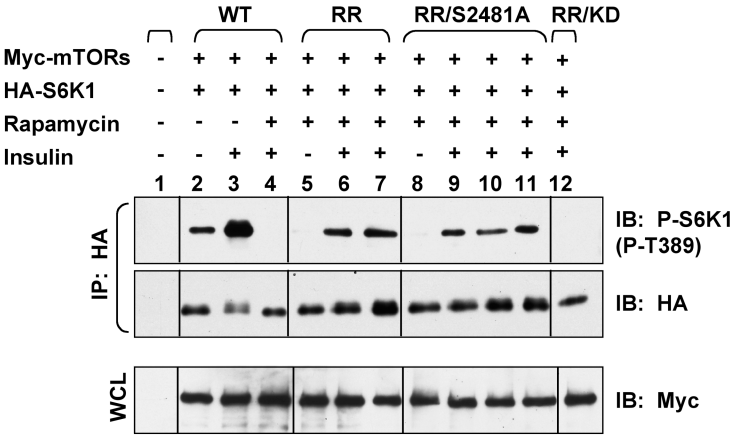

Figure S3

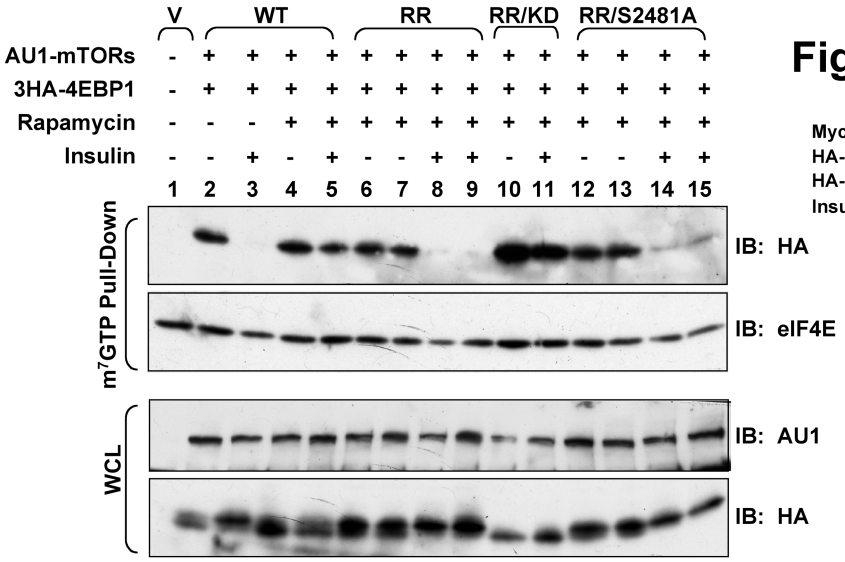

Figure S4

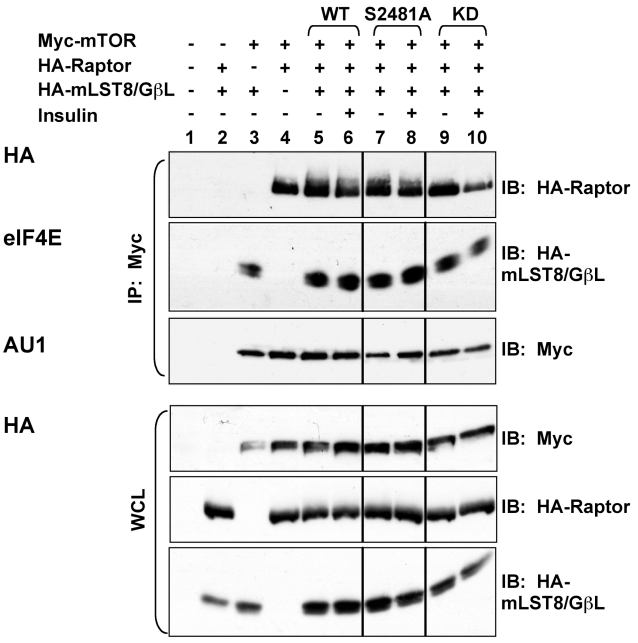

Figure S5