Supplemental Materials

Materials and methods

Cell culture and transfection

HEK293, 293T, 293 Phoenix, Hela, HepG2, and PC3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) with 50 U/mL penicillin/streptomycin. Rictor +/+ and -/- MEFs were isolated from E10.5-11.5 embryos collected from Rictor +/- intercrosses (Yang et al, 2006) and cultured in DMEM with 15% FBS. Sin1-/- MEFs were kindly provided by Bing Su (Yale School of Medicine) (Jacinto et al, 2006). Transient transfection of all cell types was performed using Lipofectamine reagent (Invitrogen). Retovirus-mediated infection was performed as previously described (Corradetti et al, 2004). For lentivirus-mediated shRNA infection, virus-containing supernatants were used to infect to target cells and the cells were selected by puromycin to obtain stable shRNA expressing cells (Sarbassov et al, 2005).

Immunostaining

Cells were fixed for 10 min by pure methanol at -20 °C. After blocking with 20 % of fetal bovine serum in PBS for 1 hr, the cells were incubated with 3 μg/mL of anti-HA mouse monoclonal antibody and 1:100 anti-ubiquitin protein conjugates rabbit polyclonal antibody in blocking reagent overnight at 4 °C, then further incubated with Alexa Fluor®594 goat anti-mouse IgG and Alexa Fluor®488 goat anti-rabbit IgG. The signals were visualized under confocal fluorescence microscope (Olympus IX SLA).

Supplementary References

Corradetti MN, Inoki K, Bardeesy N, DePinho RA, Guan KL (2004) Regulation of the TSC pathway by LKB1: evidence of a molecular link between tuberous sclerosis complex and Peutz-Jeghers syndrome. *Genes Dev* **18**(13)**:** 1533-1538

Jacinto E, Facchinetti V, Liu D, Soto N, Wei S, Jung SY, Huang Q, Qin J, Su B (2006) SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. *Cell* **127**(1)**:** 125-137

Sarbassov DD, Guertin DA, Ali SM, Sabatini DM (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* **307**(5712)**:** 1098-1101

Yang Q, Inoki K, Ikenoue T, Guan KL (2006) Identification of Sin1 as an essential TORC2 component required for complex formation and kinase activity. *Genes Dev* **20**(20)**:** 2820-2832

Supplemental Figure Legends

Figure S1 Effect of Rictor ablation on PKC and Akt phosphorylation and protein levels

Phosphorylation of the TM and HM in PKCα and Akt is eliminated in Rictor-/- MEF. Rictor +/+ and -/- MEFs were isolated from E10.5 embryos and cultured under normal conditions (medium), serum starved for 12 hr (starved), or stimulated with 10% serum for 30 min (serum). Cells were harvested and cell lysates were analyzed by immunoblotting using indicated antibodies.

Figure S2 Effect of various inhibitors on PKCα and Akt phosphorylation

A. Acute inhibition of PI3K and mTOR by LY294002 has no effect on phosphorylation of Akt TM and PKCα TM and HM. Rictor1+/+ and -/- MEF1 cells were treated with 20 nM rapamycin (R), 100 μM LY294002 (LY), and 50 nM wortmannin (W) for 1 hr. Cells were harvested and analyzed by immunoblotting using indicated antibodies.

B. Effect of prolonged treatment of PI3K/mTOR inhibitors on PKCα and Akt phosphorylation. MEF and Hela cells were treated with PI-103 (10 μM) and LY294002 (100 μM) for 24 hr as indicated. Immunoblottings were performed using indicated antibodies.

C. Effect of proline-directed protein kinase inhibitors on PKCα and Akt phosphorylation. Hela cells were treated with the following inhibitors for 24 hr, 20 nM rapamycin (R, mTORC1 inhibitor), 100 μM LY294002 (LY, PI3K and mTOR inhibitor), 20 μM U0126 (U, MEK inhibitor), 20 μM JNK inhibitor II (J), 20 μM SB203580 (SB,

p38 inhibitor), 5 μM BIM (B, PKC inhibitor), 50 nM Staurosporine (St, inhibitor of PKA and others), 20 mM LiCl (Li, GSK3 inhibitor), 400 nM Roscovitine (Ro, CDK inhibitor), 100 μM Casein kinase II inhibitor I (C).

D. Effect of rapamycin treatment on TM and HM phosphorylation in PKCα and Akt in A549 cells. A549 cells were treated with 100 nM rapamycin for indicated times. Total cell lysates were probed with PKC and Akt antibodies as indicated.

E. Knockdown of mTOR combined with rapamycin treatment does not affect Erk and PKCδ phosphorylation. Hela cells with mTOR knockdown using shRNA-containing lentivirus were pretreated with 100 nM rapamycin for 24 hr. Immunoblottings were performed using indicated antibodies.

Figure S3 PKCα interacts with Rictor but not Raptor

HEK293 cells were transfected with HA-PKCα and Myc-Rictor, Myc-Raptor, or empty vector as indicated. Cell lysates were immunoprecipitated with Myc antibody, and then the co-immunoprecipitated HA-PKCα was determined by HA immunoblotting. SE and LE denote short and long exposure, respectively.

Figure S4 Inhibition of Hsp90 destabilizes PKCα and PKCε in Rictor-/- cells

Rictor+/+ and -/- MEF cells were treated with 1 μM 17-AAG, a derivative of the antibiotic geldanamycin, to interfere the binding between Hsp90 and client proteins. Protein levels of PKCs were determined by immunoblotting.

Figure S5 Effect of PMA on PKC phosphorylation and activation in Sin1-/- cells

A. The PMA-stimulated MARCKS phosphorylation is truncated in Sin1-/- cells. Sin1+/+ and -/- cells were treated with PMA for indicated time (hour). Phosphorylation of MARCKS was detected by a phospho-MARCKS antibody. Protein levels of PKCα were also determined.

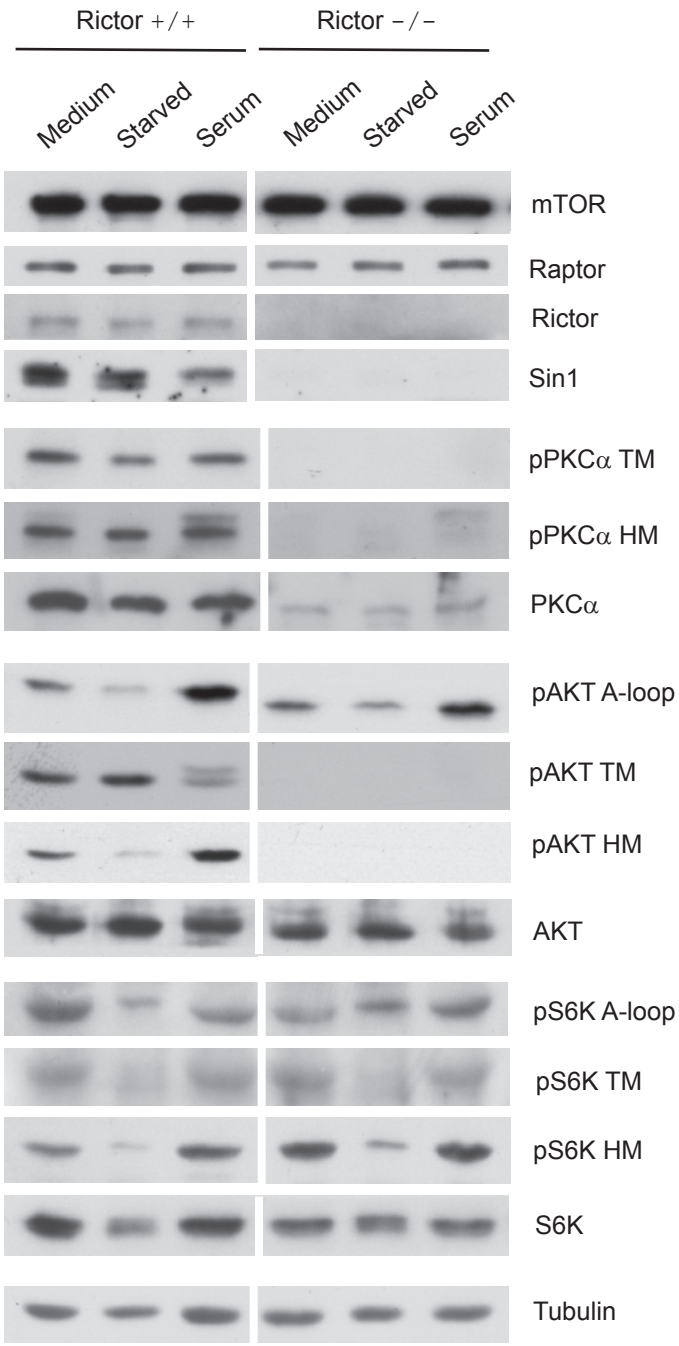
B. Lack of PMA-induced PKCα downregulation in Sin1-/- cells. Sin1+/+ and -/- MEFs were treated with PMA in the presence or absence of 5 μM MG132 for indicated time. Protein levels of PKCα were determined along with Hsp90 as a loading control. Nine-hour treatment with PMA downregulated PKCα in Sin1+/+ but not -/- cells. MG132 blocked PKCα downregulation. PMA treatments caused a downward shift of PKCα in the Sin-/- cells although the protein was not degraded.

Figure S6 A proposed model for PKCα regulation by phosphorylation

In Rictor and Sin1+/+ cells, PKCα is highly phosphorylated on A-loop (T497), TM (T638), and HM (S657). The phosphorylated PKCα is stable and ready for activation by second messengers. TM phosphorylation is completely dependent on mTORC2 possibly via a putative TM kinase (denoted by TMK). HM phosphorylation is largely dependent on mTORC2 via a putative HM kinase (denoted by HMK) or autophosphorylation, while PDK1 also affects HM phosphorylation via HMK or autophosphorylation. We speculate that mTORC2 could be a candidate for TMK and/or HMK. In Rictor-/- or Sin1-/- cells, phosphorylation on PKCα TM and HM is abolished and reduced, respectively. A-loop is also hypophosphorylated in these cells. Most of PKCα proteins accumulate in detergent-insoluble fraction and then are degraded via ubiquitin/proteasome pathway, while only small proportion of the hypophosphorylated PKCα protein is protected by Hsp90 from

degradation. The hypophosphorylated PKCα is inactive and cannot be properly activated by second messengers. Dashed arrows indicate possibly indirect connections.

Figure S1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Starved
Serum
Medium
Starved
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Rictor
Sin1
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pPKCα HM
PKCα
pAKT A-loop
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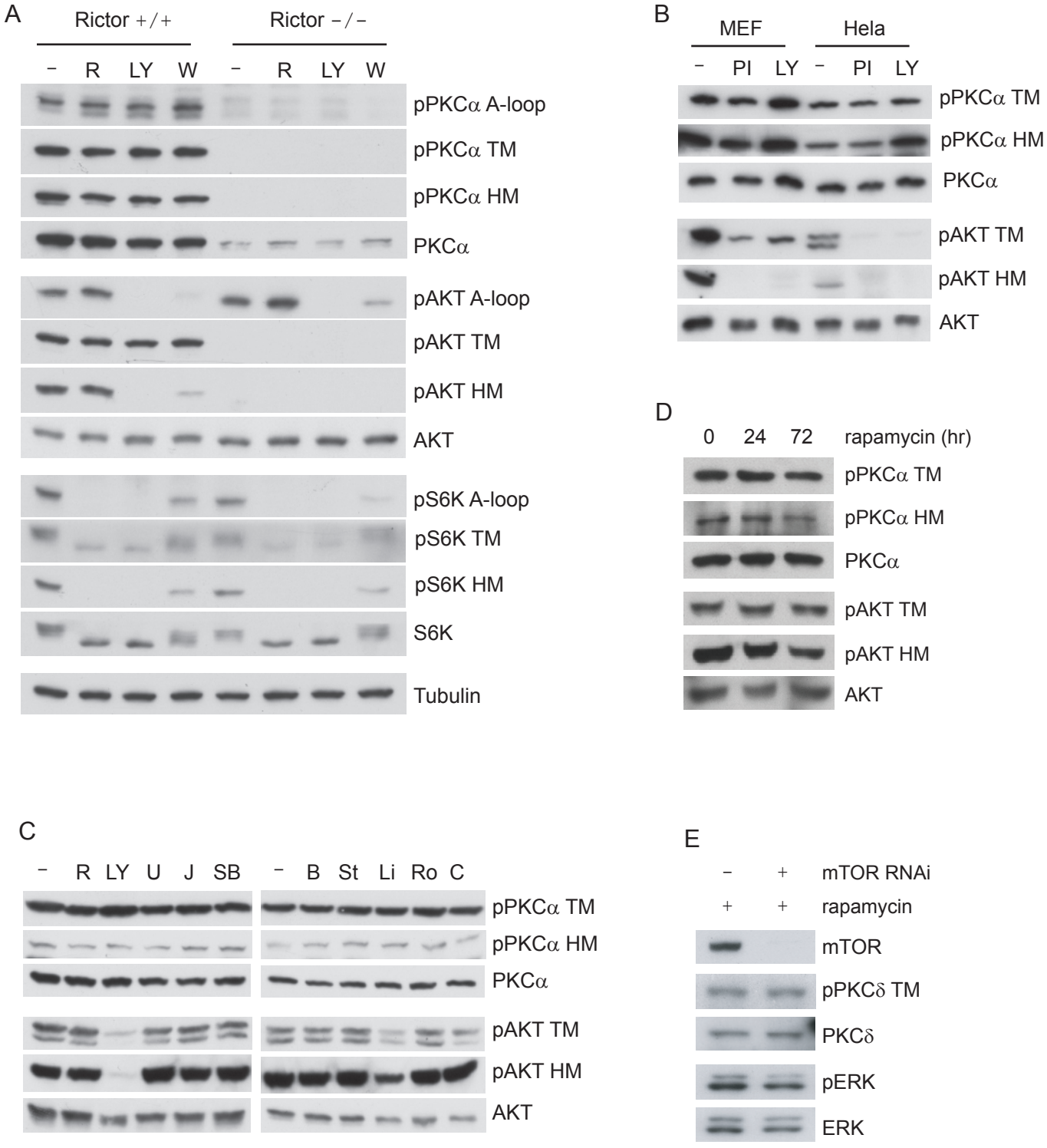
pAKT HM
AKT
pS6K A-loop
pS6K TM
pS6K HM
S6K
Tubulin

Figure S2


A
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Rictor −/−
− R LY W − R LY W
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pPKCα TM
pPKCα HM
PKCα
pAKT A-loop
pAKT TM
pAKT HM
AKT
pS6K A-loop
pS6K TM
pS6K HM
S6K
Tubulin
B
MEF
Hela
− PI LY − PI LY
pPKCα TM
pPKCα HM
PKCα
pAKT TM
pAKT HM
AKT
D
0 24 72 rapamycin (hr)
pPKCα TM
pPKCα HM
PKCα
pAKT TM
pAKT HM
AKT
C
− R LY U J SB − B St Li Ro C
pPKCα TM
pPKCα HM
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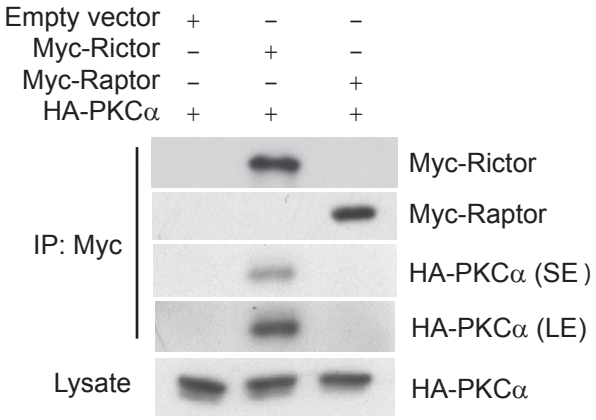
pERK
ERK

Figure S3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Myc-Rictor – + –
Myc-Raptor – – +
HA-PKCα + + +
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Myc-Raptor
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HA-PKCα (LE)
Lysate
HA-PKCα

Figure S4

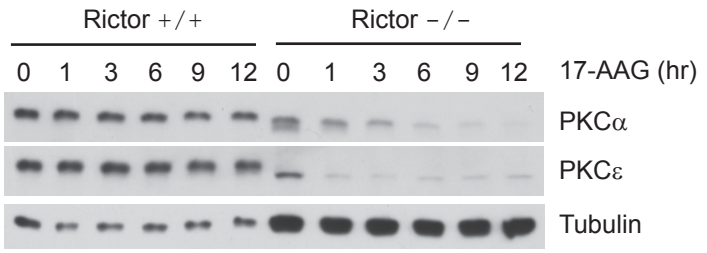
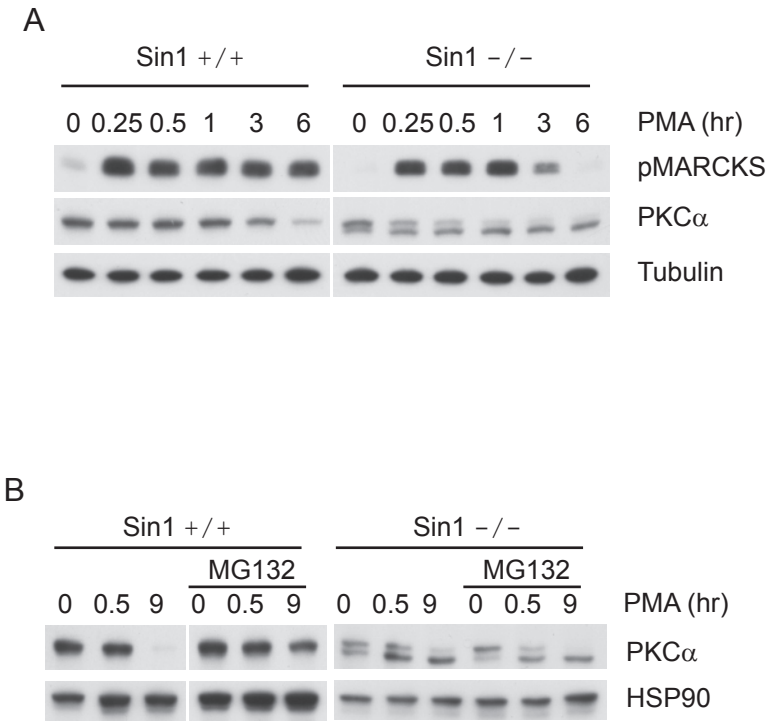


Figure S5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Sin1 +/+
Sin1 −/−
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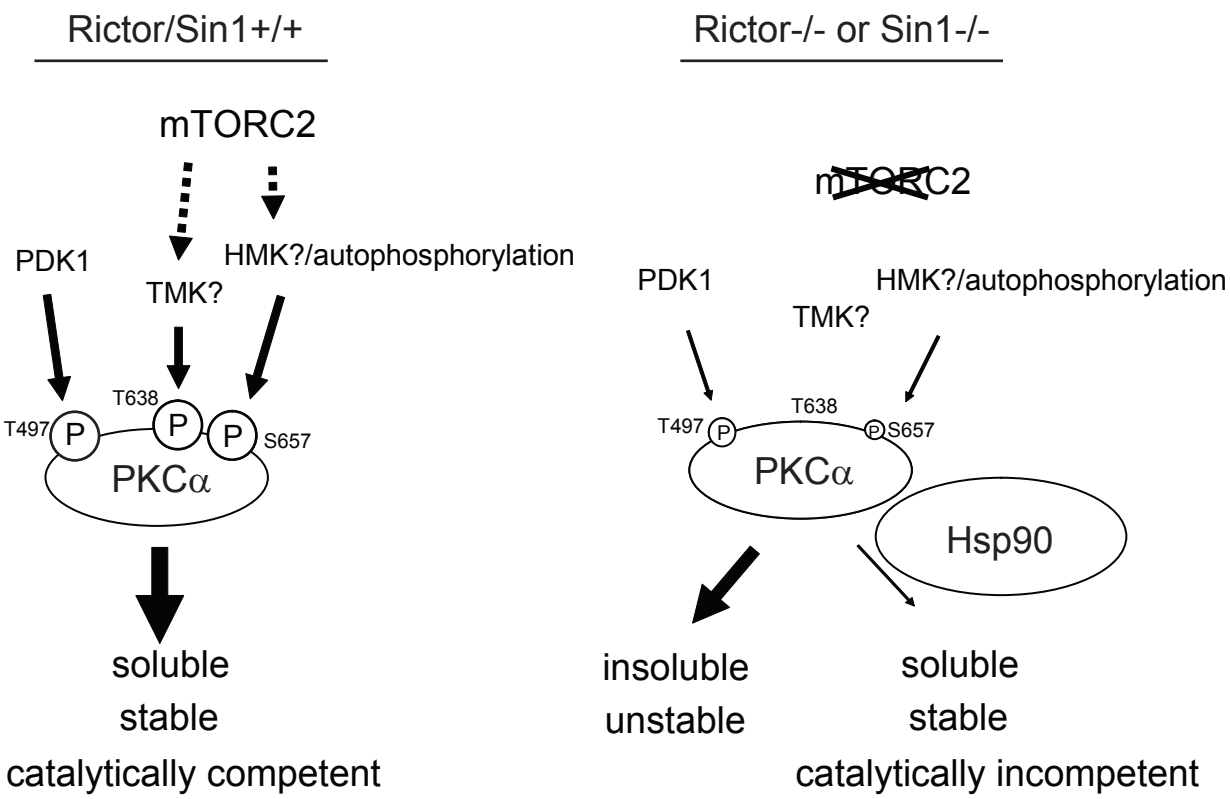
0 0.25 0.5 1 3 6
PMA (hr)
pMARCKS
PKCα
Tubulin
B
Sin1 +/+
Sin1 −/−
MG132
MG132
0 0.5 9 0 0.5 9
0 0.5 9 0 0.5 9
PMA (hr)
PKCα
HSP90

Figure S6


Rictor/Sin1+/+
mTORC2
PDK1
TMK?
HMK?/autophosphorylation
T497
T638
S657
P
PKCα
soluble
stable
catalytically competent
Rictor-/- or Sin1-/-
mTORC2
PDK1
TMK?
HMK?/autophosphorylation
T497
T638
S657
PKCα
Hsp90
insoluble
unstable
soluble
stable
catalytically incompetent