SUPPLEMENTARY INFORMATION

METHODS

Reagents

Cell-permeable peptides AP (Antennapedia, RQIKIWFQNRRMKWKK) and AP-Akt, based on human Hsp90β region (327-340aa) (RQIKIWFQNRRMKWKKLEFRALLFIPRRAP), which binds to Akt were synthesized by Keck Biotechnology Resource Laboratory (Yale University). pFA6a-13myc-PKC1 plasmid (pHPS75) was obtained from H.P. Schmitz (Univ. Osnabrueck) (Jacoby et al., 1997). 17-AAG was obtained from Alexis Biochemicals (Lausanne, CH). MG132, and wortmannin were obtained from Sigma (St. Louis, MO). Rapamycin was obtained from LC Laboratories (Woburn, MA). PKCα monoclonal antibody was from Upstate (Charlottesville, VA) and hsp90 monoclonal ab was from BD Bioscience (San Jose, CA). HA and Myc antibodies were purchased from Covance (Berkeley, CA), GFP antibody was from Abcam (Cambridge, MA). Tubulin and actin antibodies were from Sigma and ERK2 antibody from Santa Cruz Biotechnology (Santa Cruz, CA). SIN1 K87 and mTOR 26E3 and N5D11 antibodies were previously described (Cheng et al., 2005) (Jacinto et al., 2006; Jacinto et al., 2004), rictor antibody was obtained from Bethyl Laboratories (Montgomery, TX). All other antibodies were from Cell Signaling Technology (Beverly, MA).

Yeast culture and depletion experiments

PCR cassettes were used to generate GAL1p-HA-AVO1 and GAL1p-HA-KOG1 and transformed into TB50 strain. Yeast strains (Supplementary Table 1) were transformed as previously described (Jacinto et al., 2001). For AVO1 and KOG1
depletion experiments, yeast cells from logarithmically growing SGal cultures were inoculated into SD medium and grown at 30°C at different time points before harvest and lysis. Cells were lysed by mechanical disruption as described previously (Jacinto et al., 2001).

**Drosophila RNA interference experiments.**

*Drosophila* RNA interference experiments were performed as described (Yang et al., 2006) using the following primers:

dRaptor forward primer:
5’ttatatcactcactataggagaGTCCTGAGCCTCTAATCCGCAAGA

dRaptor reverse primer:
5’ttatatcactcactataggagaGGCCAGAGTTGCCGTCTTCATGC

dSIN forward primer:
5’ttatcacgtcactataggagaGAGCCGGATCGCAACTAC

dSIN reverse primer:
5’ttatcacgtcactataggagaGGCGTCGCTCCAAAATCTCG

The lower case region indicates the T7 promoter sequence.

**Preparation of retrovirus and infection of MEF cells**

The pMIGW retroviral vectors containing HA-SIN1, HA-Akt and its derived mutants were co-transfected with the retroviral packaging vectors containing the sequence for MoMLV gag-pol and the sequence for VSVg envelope into 293T cells by a calcium phosphate based protocol. Supernatants containing the retrovirus were collected 48 and 72 hours later. MEFs were incubated with virus containing medium in the
presence of polybrene (hexadimethrine bromide, Sigma) during centrifugation at 1000Xg for 1 h at 32°C. Stable cell lines were established by fluorescence activated cell sorting (FACS) and analyzed 7 days after infection.

Adenovirus expressing GFP (Ad-GFP) or a dominant negative HA-Hsp90β (Ad-Hsp90β−DN) were described before (Miao et al., 2008). MEFs were incubated with 100 MOI of adenovirus for 6 hours before replacing the culture medium. Infected cells were harvested and analyzed 48 h after infection.

**Preparation and dephosphorylation of GST-Akt1**

GST-Akt1 was expressed in HEK293T cells by transient transfection with lipofectamine 2000 (Invitrogen). Two days after transfection, cells were treated with 50 µM of Ly294002 for two hours before harvest and lysis in ice-cold lysis buffer [50 mM Tris-HCl pH 7.4, 150mM NaCl, 1% Triton X-100 and EDTA-free protease inhibitors (Roche)]. After clearing by centrifugation at 13,000Xg for 15 min, GST-Akt1 was immobilized on glutathione-agarose beads and treated with lambda-PPase (NEB) for 90 min at 30°C, then extensively washed with lysis buffer containing phosphatase inhibitors (2mM EDTA, 10 mM pyrophosphate, 0.5 mM orthovanadate, 10 mM glycerophosphate) before elution by incubation with the GST elution buffer (100 mM Tris-HCl pH 7.5, 100 mM NaCl, 5% glycerol) containing 40 mM reduced-glutathione.

**Kinase assay**

Immunoprecipitation of mTOR complexes and kinase assays were performed as previously described (Sarbassov et al., 2005) with minor modifications. The mTOR complexes were immunoprecipitated from HeLa cells that were lysed in CHAPS Lysis
Buffer [40 mM Hepes pH 7.5, 120 mM NaCl, 0.3 % CHAPS, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 0.5 mM orthovanadate, and EDTA-free protease inhibitors (Roche)]. 3 µg of anti-SIN1 Ab (Cheng et al., 2005) was added to the clarified lysates and incubated with rotation for 2 hours, followed by addition of 25 µl of protein G-agarose (Roche) and incubation for an additional hour. Immunoprecipitation of HA-mTOR was performed by incubating the lysates from HeLa cells transiently transfected with HA-mTOR constructs with 25 µl of monoclonal anti-HA agarose conjugate (Sigma) for 2 hours. Protein G-agarose and HA-agarose binding immunoprecipitated were washed 3 times with CHAPS lysis buffer and once with mTORC2 kinase buffer (25mM Hepes pH7.5, 100 mM potassium acetate, 2mM MgCl₂).

For kinase assay reactions, immunoprecipitates were incubated in a final volume of 30 µl for 30 min at 37 °C in the mTORC2 kinase buffer containing 500 ng of GST-Akt previously dephosphorylated and 500 µM ATP. The reaction was stopped by adding 200 µl of Enzyme Dilution buffer (20 mM MOPS pH 7.0, 1 mM EDTA, 0.3% CHAPS, 5% glycerol, 0.1% 2-mercaptoethanol) and the supernatant was separated from the protein G-agarose by centrifugation. Substrate-containing supernatant and immunocomplex-containing protein G-agarose fractions were fractionated by SDS-PAGE and analyzed by immunoblotting.

REFERENCES


**Supplementary Figure 1.** The anti-phospho-PKCα/βII Thr638/641 antibody detects phosphorylation at the turn motif of both PKC and Akt. **A.** Wild type and SIN1−/− MEFs were grown and stimulated as described in Figure 1A. Total cellular extracts were prepared for immunoblot analysis using anti-phospho-PKCα/βII Thr638/641 antibody (top panel) and anti-Akt antibody (bottom panel). The arrow indicates the phospho-PKCα/βII at Thr638/641 (p-PKCα/βII TM). The arrowhead shows a 60 kDa protein that cross-reacts with the anti-phospho-PKCα/βII T638/641.

**B.** Wild-type or SIN1−/− MEFs were infected with either an empty virus (−), or with a HA-Akt expression virus (+). HA-Akt was immunoprecipitated with an anti-HA antibody and analyzed by immunoblotting using an anti-phospho-PKCα/βII Thr638/641 (top panel) and an anti-HA antibody (bottom panel). The TM site phosphorylated Akt (p-Akt TM) and HA-Akt are indicated.
Supplementary Figure 2. Prolonged rapamycin treatment diminishes TM site phosphorylation in PC3 and Jurkat cell lines. PC3 (A), Jurkat (B), HEK293T (C), and HeLa (D) cells were either untreated (time 0), or incubated with rapamycin (100 nM) for 24, 48, and 72 h before harvesting. SIN1 was immunoprecipitated and associated mTOR and rictor were detected by immunoblotting. Total extracts were analyzed by immunoblotting for phosphorylation at the TM and HM sites and for Akt and PKCα protein levels.
Supplementary Figure 3. The TORC2 components are essential for *Saccharomyces cerevisiae* PKC1 and *Drosophila* dAkt TM phosphorylation.

A. The yeast TORC2 component AVO1, but not the TORC1 component KOG1, is required for the TM site phosphorylation of yeast PKC1. *S. cerevisiae* containing either HA-AVO1 or HA-KOG1 (under the control of the GAL promoter in the genome) were transformed with a myc-PKC1 plasmid DNA and were grown in galactose medium until log phase (lanes 1, 4). At log phase, cells were shifted to glucose medium and grown for another 6 (lanes 2, 5), or 8 (lanes 3, 6) hr to reduce the HA-AVO1 or HA-KOG1 expression before harvest. Myc-PKC1 was immunoprecipitated using an anti-myc antibody, followed by immunoblot analysis for TM site phosphorylation using anti-phospho-PKCa/βII T638/641. HA-AVO1 and HA-KOG1 levels were determined by immunoblotting with an anti-HA antibody. Actin level was used as a loading control.

B. *Drosophila* TORC2 is required for TM site phosphorylation of dAkt. *Drosophila* S2 cells were transfected with either control vector (C), or with expression vectors for *Drosophila* Raptor siRNA (dRapt), or SIN1 siRNA (dSIN1) as indicated. Transfected cells were either starved overnight or starved then restimulated with insulin for 30 min before harvest, followed by immunoblot analysis for total dAkt and phospho-dAkt at the HM and TM sites.
Supplementary Figure 4. The half-life of Akt and PKCα in SIN1−/− cells is comparable to wild type cells. Wild type or SIN1−/− MEFs were untreated or treated with cycloheximide (10mM) for different time points. Total cell extracts were analyzed by immunoblotting with an anti-Akt (left graph) or an anti-PKCα (right graph) antibody. Protein levels were quantitated using ImageQuant software and plotted using wild type, untreated (0 h) as the reference point, which was assigned a value of 1.
Supplementary Figure 5. The remaining Akt and PKCα in SIN1−/− cells are protected from degradation by the molecular chaperone Hsp90.

A. Disruption of Hsp90-Akt interaction destabilizes Akt in SIN1−/− cells but not in wild type cells. Wild type and SIN1−/− MEFs were incubated with a control Antennapedia peptide (AP), or with a peptide corresponding to human Hsp90β aa 327-340 (AP-Akt) (3 µM. Cells were harvested after incubation at different time points (h) and the protein levels of Akt, ERK2, and tubulin were determined by immunoblotting.

B. Expression of a dominant negative Hsp90 destabilizes Akt and PKCα in SIN1−/− cells but not in wild type cells. Wild type (WT) and SIN1−/− MEFs were infected with 100 MOI of adenovirus expressing either GFP alone (Ad-GFP) or a dominant negative HA-Hsp90β (Ad-Hsp90β−DN). Infected cells were harvested 48 h later, and the protein levels of Akt, PKCα, tubulin, and exogenous HA-Hsp90β were determined by immunoblotting.
Supplementary Figure 6. Heat shock increases binding of Akt with Hsp90 in SIN1<sup>−/−</sup> cells. Wild type and SIN1<sup>−/−</sup> MEFs, either (A) untransfected or (B) transfected with an empty vector (V) or an HA-Akt expressing vector were grown at 37°C, then shifted to a 45°C (A) or a 42°C (B) incubator for the indicated time periods (h) before harvesting. Akt was immunoprecipitated using an anti-Akt antibody (A) or an anti-HA antibody (B), and the immunocomplex was analyzed by immunoblotting for the associated Hsp90. The endogenous Akt (A) and the transfected HA-Akt (B) were also determined by immunoblotting.
Supplementary Figure 7. Akt is degraded in SIN1⁻/⁻ cells upon Hsp90 inhibition via the proteasome pathway. Wild type and SIN1⁻/⁻ MEFs were stably infected with retroviral expression vectors for a wild type, HA-tagged Akt, or mutated HA-tagged Akt at T450A, or at T443A/T450A as indicated. Cells were treated either with vehicle (0), or with 17-AAG (1 mM) alone, or with both 17-AAG and MG132 (10 mM), for the indicated time periods (h) before harvesting. Total HA-Akt level was determined by immunoblotting using an anti-HA antibody.
Supplementary Figure 8. Mutation of the conserved basic residues in Akt1 that are predicted to interact with the TM site phosphate did not abolish Akt phosphorylation. Wild type MEFs were stably infected with a control vector (Vector) or with retroviral expression vectors for HA-Akt (WT) or methionine mutants as indicated. Extracts from each cell line were immunoprecipitated using HA antibody and further analyzed for phosphorylation and immunoprecipitated protein levels.
## Table SI. Yeast strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB50a</td>
<td>MATa leu2-3, 112 ura3-52 trp1 rme1 his3 HMLa HIS4</td>
</tr>
<tr>
<td>CL1</td>
<td>TB50a [kanMX6]-GAL1p-3HA-AVO1</td>
</tr>
<tr>
<td>CL2</td>
<td>TB50a [kanMX6]-GAL1p-3HA-KOG1</td>
</tr>
</tbody>
</table>