Supporting Information

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SI Materials and Methods

Cell Culture. Cells were seeded and grown for 48 h in DMEM supplemented with 10% (vol/vol) serum (basal conditions). Cells were starved of serum for 14 h unless noted otherwise before restimulation with 100 nM insulin (Sigma) for 15 min. Primary hepatocytes were isolated as previously described (6). For mTORC2 knockdown, HeLa cells were infected with lentiviruses carrying shControl or shRictor (TRC library; Sigma Aldrich) at a multiplicity of infection of 0.1. Cells were selected after 24 h with 1 μg/mL puromycin for 1 wk, then subcloned and kept under puromycin selection. Transfection was performed using X-tremegene HD (Roche) using 1 mL Optimem (Gibco), 6 μg plasmid DNA, and 18 μL transfection reagent per 10-cm dish. Transient siRNA-mediated knockdown was performed as described previously (7).

Protein Lysates, Fractionation, and Immunoprecipitation. Films were scanned on CanoScan 9000F and signals were quantified using densitometry functions of the FIJI application. For subfractionation, endoplasmic reticulum (ER) was isolated by isopycnic flotation using a previously described protocol (8). Preparation of crude and pure mitochondrial extracts and mitochondria-associated ER membrane (MAM) isolation were performed as previously published (9). Ribosome stripping was performed as previously published (10). Immunoprecipitation and mammalian target of rapamycin complex 2 (mTORC2) kinase assay was performed using a previously described protocol (11). Briefly, cells were lysed on the culture plates after one washing step with cold PBS. Lysates were precleared with respective protein A or G Sepharose beads for 20 min. Lysis buffer was either CHAPS IP buffer (0.3% CHAPS, 40 mM Hepes pH7.5, 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glyerophosphate, HALT phosphatase, and protease inhibitor; Promega) or RIPA buffer (1% Triton-X, 0.1% SDS, 0.5% deoxycholate, 50 mM Tris pH 7.5, 150 mM NaCl, HALT phosphatase, and protease inhibitor; Promega), as noted, respectively. Control/mock IP was performed using equal amount of mouse/rabbit or goat IgG, respectively. mTOR complex IPs were performed for 4 h with IP antibody, followed by 1 h in the presence of protein A Sepharose beads, both steps at 4 °C. Beads were washed three times in IP buffer, and loading buffer was added. IPs targeting the IP_3 receptor (IP3R) were boiled at 65 °C for 25 min instead of standard 95 °C for 5 min to prevent precipitation of the transmembrane proteins. To measure the phosphorylation status of IP3R, HeLa cells were starved for 14 h, treated with 500 nM PP242 or DMSO for 20 min and stimulated with 20% (vol/vol) FCS for 20 min. IP3R was immunoprecipitated and blots were probed with anti-IP3R or antiphospho-Akt-Substrate antibody. To measure the phosphorylation status of HK2, HeLa cells were transfected with HK2-HA 48 h before the experiment. HeLa cells were starved for 14 h, treated with 500 nM PP242 or DMSO for 20 min, and stimulated with 20% (vol/vol) FCS for 20 min. Blots were probed with anti-HA or antiphospho-Akt-Substrate antibody.

Microscopy. Cells were washed in PBS, fixed with 37 °C 4% (vol/vol) paraformaldehyde for 2 min, washed in PBS twice, treated with 0.1% TritonX 100 for 10 min at room temperature, and blocked in 3% (wt/vol) BSA for 1 h at room temperature. For immunofluorescence, primary antibodies were added over night at 4 °C (1:50 for anti-rictor, 1:200 for anti-HSP47), washed three times in PBS at room temperature, and incubated with secondary antibodies (anti-rabbit Alexa647 1:200, anti-mouse Alexa488 1:200) for 2 h at room temperature. Slides were washed three times in PBS and mounted in Mowiol mounting medium [2.4 g Mowiol 4-88; Calbiochem; 6 g glycerol, 6 mL water, 12 mL 0.2 M Trix pH8.5, 2.5% (wt/vol) (1,4,-diazobicycli-[2.2.2]-octane); Sigma-Aldrich]. Fluorescence was measured on LSM510 or LSM710. Images analyzing rictor localization or ER/mitochondrial colocalization were acquired using 3D-deconvoluted stacks using the Huygens application. Colocalization (Pearson's correlation coefficient) was measured with Imaris application using automatic thresholding.

For electron microscopy, mouse liver was cut into small pieces of 1–2 mm and fixed with 0.1 M phosphate buffer containing 3% (wt/vol) formaldehyde and 0.3% glutaraldehyde for 30 min at room temperature and with fresh fixative overnight at 4 °C. Then samples were washed with PBS three times for 30 min. Dehydratation was done at 4 °C in 50%, 70%, 90% (vol/vol) methanol/PBS each for 1 h. Infiltration with LR gold was done according to the manufacturer's instructions (LR gold, London Resin). Polymerization was done at –10 °C for 24 h. Sections of 75 nm were collected on Formvar/carbon-coated Ni-grids. Sections were stained for 15 min with 4% (wt/vol) uranyl-acetate and contrasted with lead-citrate for 60 s, visualized by a Philips EM100 electron microscope. Images were cropped for individual hepatocytes using Adobe Photoshop. Cell parameters, including ER length and mitochondrial size, were calculated using FIJI application.

Chemicals. Antibodies: mTOR, Akt, Akt pS473, Akt pT308, S6, PDI, insulin receptor, Lamp1, rictor IB, voltage-dependent anion-selective channel 1 (VDAC1), IP3R (=pan IP3R antibody), Grp75, phosphoinositide-dependent protein kinase-1, Rheb, HA, mitofusin 2 (Mfn2), RXRXXpS/T (9611), Hexokinase 2 (HK2), CoxI, cleaved Parp from Cell Signaling; Sin1, raptor, Rpl26 from Bethyl; PGK1, acyl CoA synthetase 4 (ACSL4), IP3R3 from Santa Cruz; phosphatidyl ethanolamine methyltransferase (PEMT2), rictor IF from Protein Atlas; Rab32 from Sigma; calnexin from BD Bioscience; CoxIV from Invitrogen; Hsp47 from Enzo Life Science; rictor EM from Abcam (ab104838); Rab5 antibody was obtained from Martin Spiess; rictor IP antibody was obtained from Markus Rüegg. Chemicals used were PP242 (Chemdea); insulin, ionomycin, thapsigargin and arachidonic acid (Sigma); Fura2-AM (Invitrogen).

Metabolic Measurements. Mitochondrial potential measurements were performed using MitoPT-TMRM (tetramethylrhodamine, methyl ester; ImmunoChemistry Technologies) following the manufacturers' instructions on a Bio-Rad Biomek NK; statistical analysis was performed using FlowJo 9.4. For mitochondrial Ca^{2+} measurements, mouse embryonic fibroblasts (MEFs) were transiently transfected with the ratiometric cameleon probe 4mtD3cpv targeted to the mitochondrial matrix 48 h before the experiments. Ratiometric images of Ca^{2+} signals were obtained using a microscope (Axio Observer, Zeiss) equipped with a Lambda DG4 illumination system (Sutter Instrument). Cells were illuminated at 440 nm (440AF21; Omega Optical) through a 455DRLP dichroic mirror, and emission was collected alternatively at 480 nm (480BP10; Omega Optical) and 535 nm (535AF26; Omega Optical), using a cooled, 12-bit CCD camera (CoolSnap HQ; Ropper Scientific). Image acquisition and analysis were performed with the Metafluor 6.3 software (Universal Imaging). Experiments were performed at room temperature in Hepes-buffered solution containing: 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 Hepes, 10 mM glucose,

pH-adjusted at 7.45 with NaOH. For intracellular Ca^{2+} measurements, cells were plated 48 h before experiment on Lab-Tek chamber slides (ThermoFisher). Chambers were washed loaded for 30 min with HBSS plus calcium, 10% (vol/vol) FCS, and 1 μM Fura2-AM (Invitrogen). Ten minutes before measurement, the medium was replaced by adding calcium-free HBSS (Sigma) and the Fura2 signal was measured as described previously (12). The signal was captured and processed on Zeiss Cell Observer light microscope. Area under the curve (AUC) was calculated with Graphpad Prism 5 using baseline correction. Stimulations were performed with 200 μM ATP, 10 μM thapsigargin (TG), 80 μM arachidonic acid, or 10 μM ionomycin. For apoptosis measurements, Annexin V Alexa Fluor 488 (Invitrogen) measurements were performed as previously described (13). ATP was quantified using CellTiter-Glo Luminescent assay (Promega).

Statistics. Student's *t* test was calculated using QuickTTestX 1.0 application.

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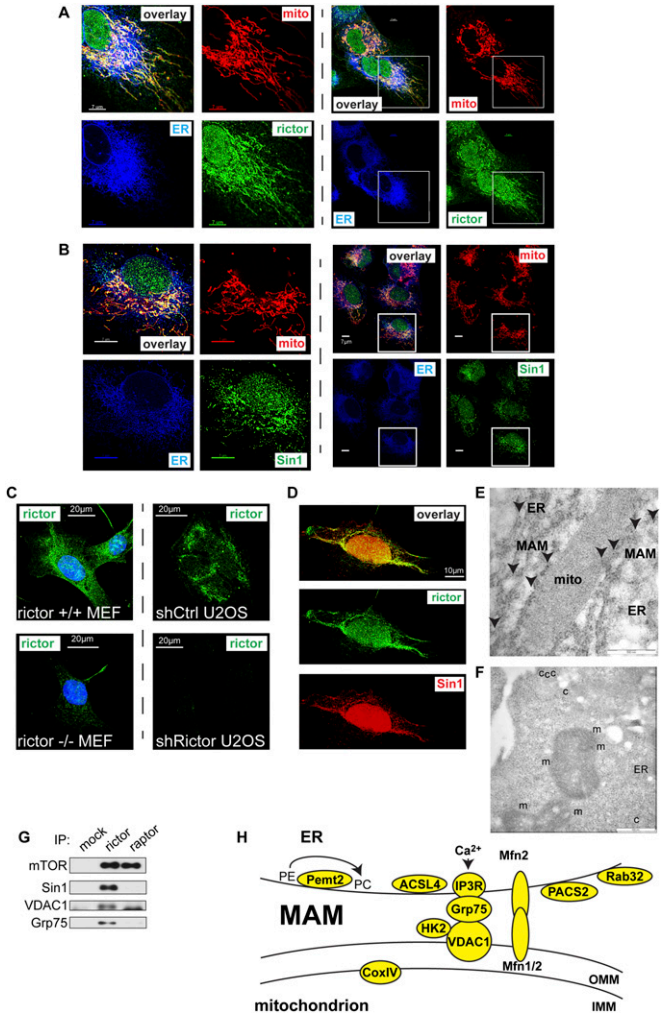

Fig. S1. (*A*) Individual channels from Fig. 1*C*, showing ER (blue, ER-GFP, BACMAM 2; Invitrogen), mitochondria (red, Mito-RFP, BACMAM 2; Invitrogen), and endogenous rictor (green) in U2OS cells. (Scale bar, 7 μm.) Shown is maximum-intensity projection of 3D-deconvoluted, confocal image stack. (*Left*) Zoomed-in view from *Right*. (*B*) Immunofluorescent image showing ER (blue, ER-GFP), mitochondria (red, Mito-RFP), and endogenous Sin1 (green) in U2OS cells. (Scale bar, 7 μm.) Cells were grown on coverslips in normal medium before PFA fixation. (*C*) Control for specificity of the rictor antibody. Rictor was stained as previously in inducible rictor KO and control MEFs (*Left*, counterstained with DAPI) or in U2OS cells with a stable rictor knockdown (*Right*). Acquisition settings for image pairs are equal. (Scale bar, 20 μm.) Cells were grown on coverslips in normal medium before PFA fixation. (*D*) Immunofluorescent image showing overlap of endogenous rictor (green) and endogenous Sin1 (red) in U2OS cells. (Scale bar, 10 μm.) Cells were grown on coverslips in normal medium before PFA fixation. (*E*) Electron micrograph of wild-type mouse liver slices staining for rictor (arrowheads). Mito, mitochondrion. (Scale bar, 500 nm.) Mice were fed a standard chow diet and killed in the morning. (*F*) Electron micrograph of HEK293T cells staining for rictor; m, rictor in proximity to mitochondrion; c, rictor in cytosol or attached to unidentified organelle. (Scale bar, 500 nm.) Cells were grown in normal medium before fixation. Unlike in liver cells, the ER is not clearly visible in HEK cells. Forty-eight percent of rictor was cytosolic, 38% associated with mitochondria, 11% in close proximity with the plasma membrane, and 4% was nuclear ($n = 7$). (*G*) mTORC2 (rictor) but not mTORC1 (raptor) coimmunoprecipitates the MAM tether VDAC1-Grp75. Mouse liver extracts were used as starting material for IP. Lower band in VDAC1 panel represents signal from light antibody chain. (*H*) Schematic overview of MAM including marker proteins used in this study.

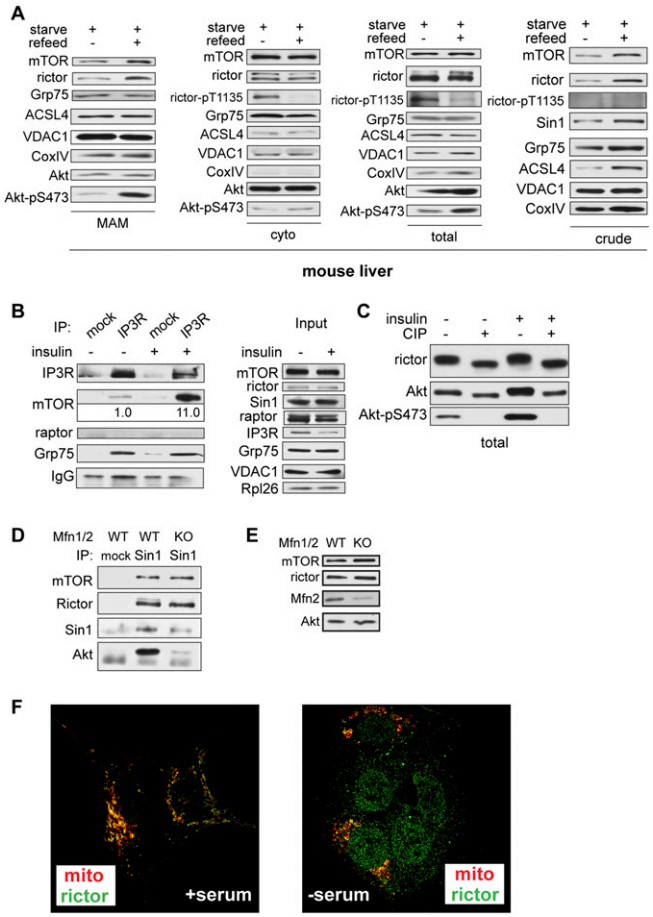

Fig. S2. (*A*) mTORC2 localization to MAM is increased in livers of refed control mice, indicating that increased mTORC2 localization to MAM is also observed under physiological stimulation by food intake. Mice were starved for 14 h and refed standard diet for 2 h. Shown are MAM, cytoplasmic, crude mitochondrial and total extracts. Rictor-pT1135 was not detected in MAM fraction and is thus not shown. Equal amount of total proteins are loaded in each lane. (*B*, *Left*) mTORC2-IP3R-Grp75 interaction is increased in total liver extracts from mice that were starved for 14 h and injected intraperitoneally with 4.5 mg/kg insulin or saline 30 min before being killed. Proteins were quantified relative to starved state. (*Right*) Total protein levels for Fig. 2*D* and panel *B*. (*C*) Rictor is hyperphosphorylated. Total proteins were extracted from mouse livers of mice that were starved for 14 h and injected intraperitoneally with 4.5 mg/kg insulin or saline 30 min before being killed. Lysates were treated with calf intestinal phosphatase (CIP; New England Biolabs) following the manufacturer's instructions. Note the band shift upon CIP treatment in rictor and Akt. (*D*) Immunoprecipitation of mTORC2 from total lysates of control and MAM-deficient Mfn1/2 KO MEFs. Cells were growing in normal medium before harvest and IP was performed as done previously in CHAPS IP buffer. Note that mTORC2 substrate Akt binding to mTORC2 is reduced in MAM-deficient cells, indicating that MAM is a major site of mTORC2 activity. (*E*) Total lysates of control and MAM-deficient Mfn1/2 KO MEFs. (*F*) Colocalization of rictor (green) and mitochondria (mito-RFP, red, BACMAM 2; Invitrogen), showing a diffuse localization for endogenous rictor (nucleus) upon serum starvation in U2OS cells. Quantification shown in Fig. 2*E*. Cells were growing on coverslips in presence or absence (14 h) of serum before fixation. (Magnification: *F*, 630×.)

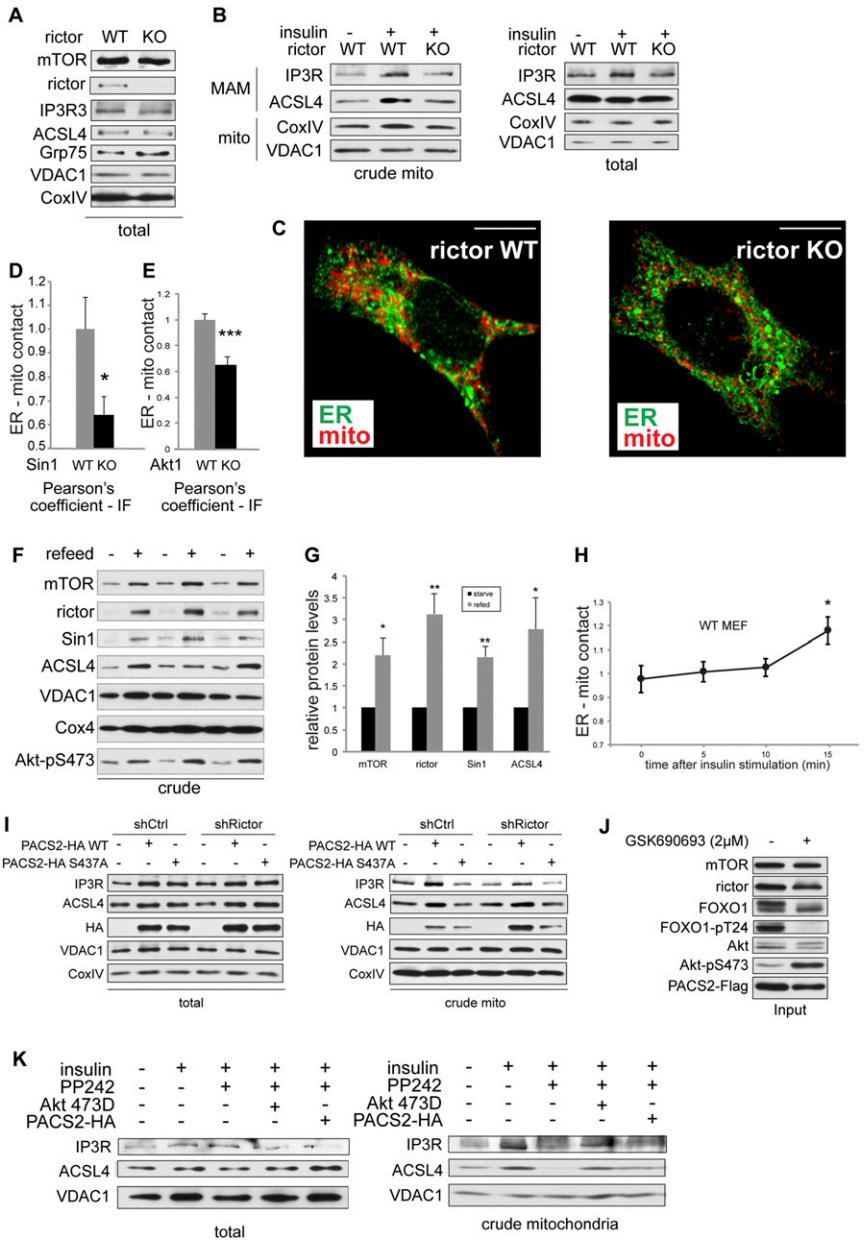

Fig. S3. (*A*) Total lysate fraction from Fig. 3*A*. (*B*) Total and crude mitochondrial extracts from livers of rictor KO or control littermate mice that were starved for 14 h and injected intraperitoneally with 4.5 mg/kg insulin or saline 30 min before being killed, showing mTORC2-dependent, insulin-stimulated accumulation of MAM marker proteins IP3R and ACSL4 in crude mitochondrial extracts. (*C*) Maximum intensity proection of representative confocal image stacks of rictor KO MEFs and control cells used for quantification of MAM in Fig. 3*D*. Cells expressed ER-GFP and mito-RFP (BACMAM 2; Invitrogen). (Scale bar, 10 μm.) (*D*) Pearson's correlation coefficient quantifying ER-mitochondrial contact as previously of Sin1 KO MEFs compared with their respective control cell line. Correlation coefficient was normalized to control cells ($n = 4$–5). (*E*) Pearson's correlation coefficient quantifying ER-mitochondrial contact as previously of Akt1 KO MEFs compared with their respective control cell line. (*F*) Crude mitochondrial extract of mouse liver cells. For corresponding MAM extracts, see Fig. 2*B*. Mice were starved for 14 h and refed for 2 h before being killed. Quantified in *G*. Extracts from two mice were pooled for each purification. (*G*) Densitometric quantification of protein levels in crude mitochondrial fraction from *F*. Note that unlike for MAM extracts (Fig. 2*B*), crude mitochondrial extracts show an increase in MAM markers when normalized to the mitochondrial VDAC1. (*H*) Life cell quantification time course of MAM in a control MEF cell line showing increased MAM formation 15 min after a 100 nM insulin stimulation relative to the starved state. Significance is calculated relative to $t = 0$ after the insulin stimulation, $n = 7$. (*I*) Total and crude mitochondrial extracts of HeLa cells after control (shCtrl) or mTORC2 knockdown (shRictor). Cells were transfected with phosphofurin acidic cluster sorting protein 2 (PACS2)-WT or PACS2-S473A 48 h before harvest and grown in normal medium. (*J*) Input of *I*. Note that even though the inhibitor induces hyperphosphorylation of Akt-pS473, Akt activity is inhibited, as judged by absence of FOXO1 phosphorylation. (*K*) Total and crude mitochondrial extracts of HeLa cells that were serum-starved for 14 h and treated with insulin 100 nM 15 min, mTOR inhibitor PP242 (500 nM, 10 min pretreatment and 15 min treatment during insulin stimulation). Cells overexpressed either Akt-S473D or PACS2 where indicated. Note the increase of MAM markers in the crude mitochondrial fraction upon insulin stimulation that is blocked by PP242. Results are shown as mean ± SEM and normalized to wild-type cells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

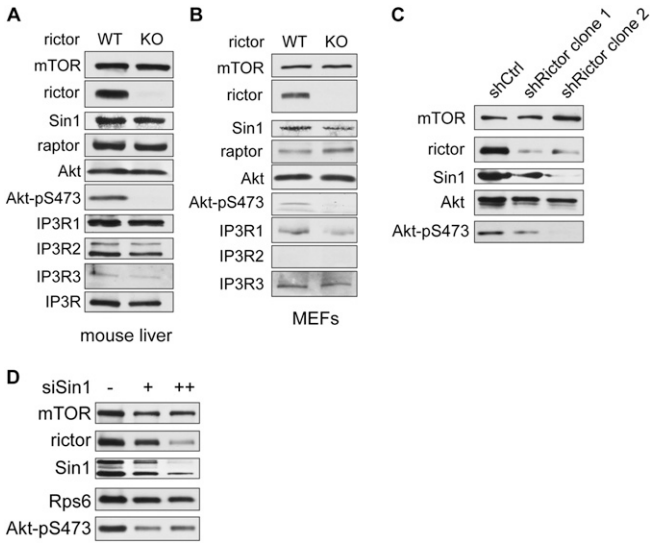

Fig. S4. (*A*) Total protein extracts from livers of a liver specific rictor KO and a control mouse showing rictor KO and reduced mTORC2 activity but no reduction in the IP3R isoforms. Note that Sin1 levels are not affected by genetic KO of rictor. Mice were starved for 14 h and injected intraperitoneally with 4.5 mg/kg insulin or saline 30 min before being killed. (*B*) Total protein extracts from MEFs where rictor KO had been induced by tamoxifen treatment and their respective controls, showing rictor KO and reduced mTORC2 activity but no reduction in the IP3R isoforms or Sin1. Cells were growing in normal medium before harvest. (*C*) Total protein extracts from stable rictor knockdown (shRictor) HeLa cells, showing reduction of Sin1 levels as previously reported (1–5). Cells were grown in normal medium and stimulated with 100 nM insulin for 15 min before harvest. (*D*) Total protein extracts from Sin1 knockdown HeLa cells, showing reduction of Sin1 levels and loss of rictor stability as previously reported. Cells were growing in normal medium 72 h after siRNA transfection and stimulated with 100 nM insulin for 15 min before harvest.

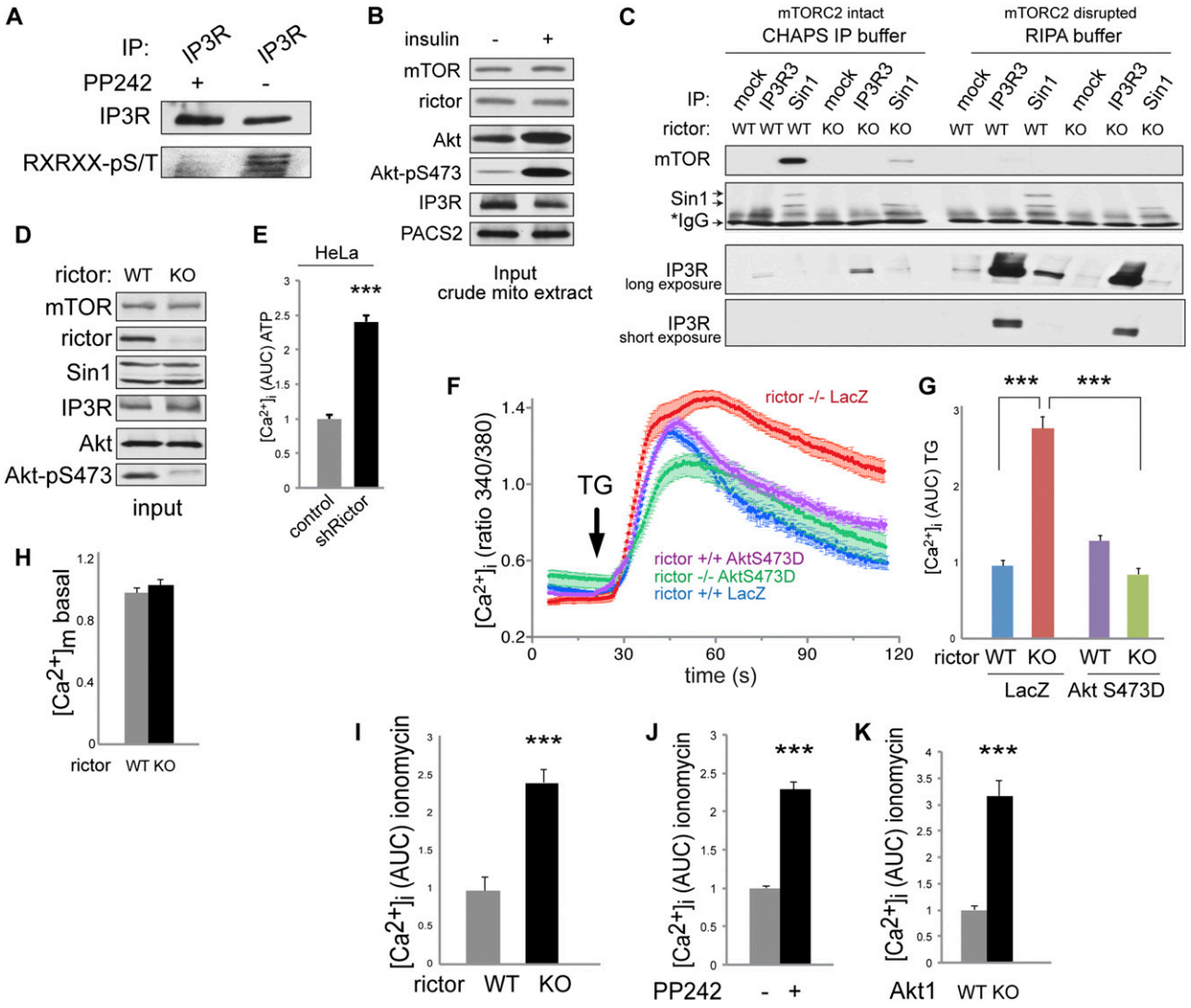

Fig. S5. (*A*) Phosphorylation of IP3R at Akt target site is sensitive to mTOR inhibition. HeLa cells were starved for 14 h, treated with 500 nM PP242 or DMSO for 20 min, and stimulated with 20% (vol/vol) FCS for 20 min. IP3R was immunoprecipitated and blots were probed with an anti-pan-IP3R or an antiphospho-Akt-Substrate (RXRXXpS/T) antibody. (*B*) Input of Fig. 4*C*. Note that starting material for IP was equalized so that equal mTORC2, IP3R, and PACS2 levels were used as input. (*C*) IP of IP3R3 and Sin1 from livers of rictor WT and KO mice. IP was performed either in CHAPS IP buffer, preserving mTORC2 integrity, or in RIPA buffer disrupting mTORC2. In CHAPS buffer, more IP3R3 can be precipitated in rictor KO vs. WT extracts because mTORC2 binding to IP3R3 appears to mask the epitope recognized by the IP antibody. Mice were fed a standard chow diet and killed in the morning. (*D*) Input of *C*. (*E*) AUC of intracellular calcium release in HeLa cells after stimulation with 200 μM ATP, quantified by the emission ratio 340/380 nm after labeling with Fura2-AM. $n = 74–104$. Cells were grown in normal medium before harvest. (*F*) Intracellular calcium release in MEFs expressing LacZ or Akt-S473D after stimulation with 10 μM TG, visualized by Fura2-AM. ($n = 16–54$). For quantification, see *G*. (*G*) AUC of *F*. (*H*) Basal (unstimulated) mitochondrial calcium concentration showing no significant difference between rictor KO and control MEFs growing in normal medium. $n = 20$. (*I*) AUC of intracellular calcium release in rictor KO and control primary hepatocytes after stimulation with 10 μM ionomycin visualized by Fura2-AM ($n = 21–33$), arbitrary units. Hepatocytes were isolated 24 h before measurement. (*J*) AUC of intracellular calcium release in HEK293T cells treated with mTOR inhibitor PP242 after stimulation with 10 μM ionomycin visualized by Fura2-AM ($n = 147–176$), arbitrary units. Cells were grown on chambered culture slides in normal medium in presence of DMSO or 1 μM PP242 for 6 h before measurement. (*K*) AUC of intracellular calcium release in Akt1 KO and control MEFs after stimulation with 10 μM ionomycin visualized by Fura2-AM ($n = 23–25$), arbitrary units. Cells were grown on chambered culture slides in normal medium. Results are shown as mean ± SEM. (*$P < 0.05$; ***$P < 0.001$.)

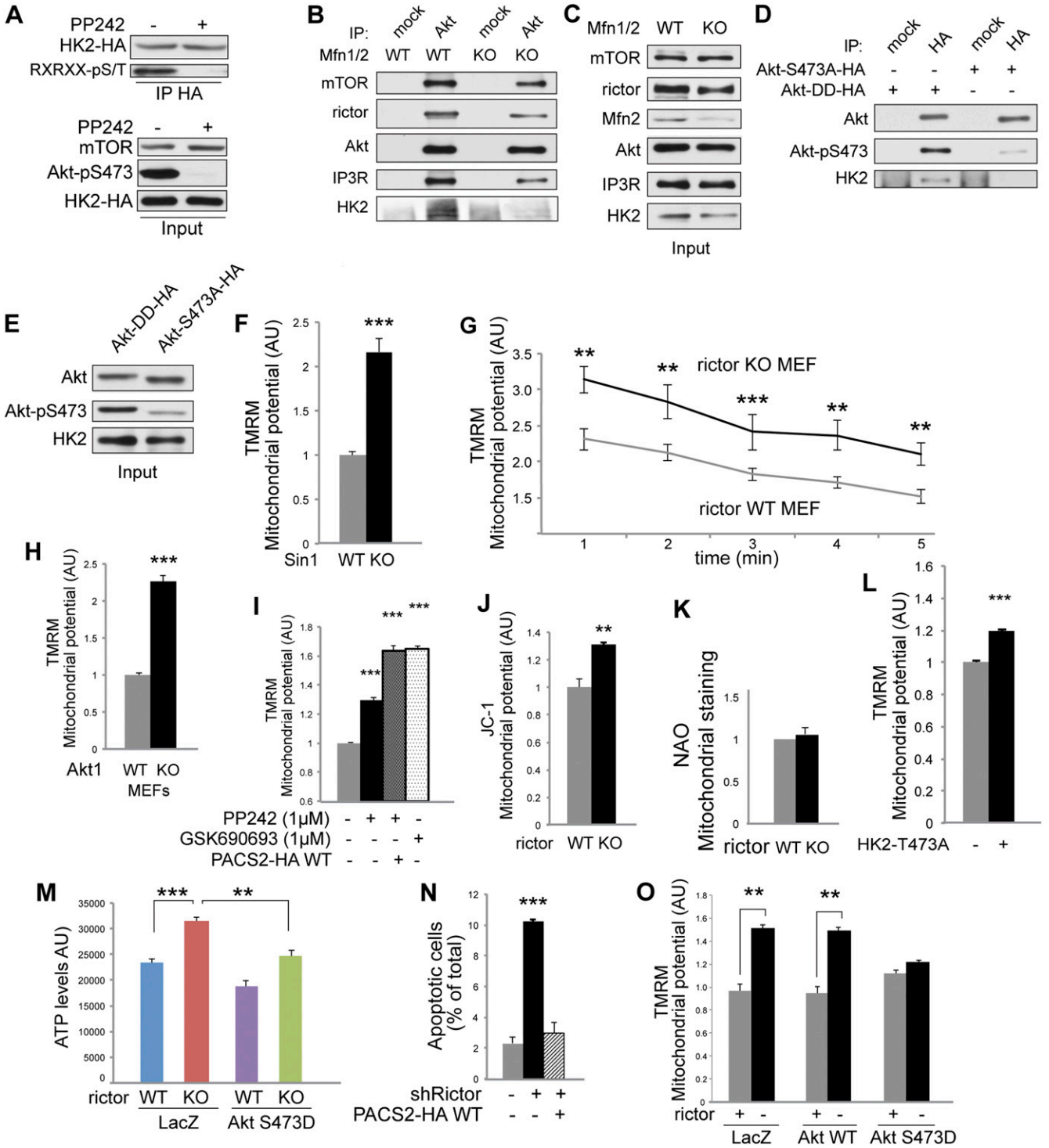

Fig. S6. (*A*) Akt-mediated phosphorylation of HK2. Overexpressed HK2-HA was immunoprecipitated from HeLa cells. Blots were probed with anti-HA or antiphospho-Akt-substrate (RXRXXpS/T) antibody. HK2-HA was immunoprecipitated and probed with a HA-tag or an Akt substrate motif antibody. Thirty-four hours after HK2 transfection, cells were serum-starved for 14 h, treated with 1 μM PP242 for DMSO for 20 min, stimulated with insulin (100 nM) for 15 min, and lysed in RIPA buffer. (*B*) IP of Akt from total protein extracts of MAM-deficient Mfn1/2 and control MEFs. Cells were grown in normal medium and lysed in RIPA buffer. (*C*) Input of *B*. (*D*) IP of overexpressed Akt mutants. Akt-DD is mutated at the sites T308D and AktS473D. Cells were growing in normal medium before lysis in RIPA buffer. (*E*) Input of *D*. (*F*) Mitochondrial potential of Sin1 KO or control MEFs, measured by TMRM intensity by life cell imaging. Arbitrary units, n = 62–69. Cells were grown on chambered slides in normal medium. (*G*) Mitochondrial potential of inducible rictor KO or control MEFs, measured by TMRM intensity by life cell imaging over 5 min. Arbitrary units, n = 17–21. Cells were grown in chambered slides in normal medium. (*H*) Mitochondrial potential of Akt1 KO and control MEFs, measured by TMRM intensity by FACS. Cells were grown in normal medium. (*I*) Mitochondrial potential of HEK293T cells, measured by TMRM intensity by FACS. Cells were serum-starved for 14 h, pretreated with mTOR inhibitor PP242 or Akt inhibitor GSK690693 for 20 min, and stimulated with insulin 100 nM for 30 min during the staining procedure. Where indicated, cells were transfected with wild-type PACS2-HA 48 h before the experiment. Note that PACS2 overexpression does not rescue the effect of mTOR inhibition. (*J*) Mitochondrial potential of inducible rictor KO or control MEFs, measured by

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JC-1 intensity by FACS. Arbitrary units, $n = 3$. Cells were grown in normal medium. (*K*) Mitochondrial staining intensity measured by a mitochondrial potential independent dye (NAO), quantified by FACS ($n = 3$). Cells were grown in normal medium. (*L*) Mitochondrial potential HeLa cells upon transfection of HK2-T473A, measured by TMRM intensity by FACS. Cells were grown in normal medium. (*M*) ATP levels of rictor KO and control MEFs infected adenovirally with LacZ or Akt-S473D, measured by CellTiter-Glo luminescence assay ($n = 12$). Cells were grown in normal medium. (*N*) Apoptotic rictor knockdown and control HeLa cells as determined by positive Annexin V staining were analyzed by FACS ($n = 3$). Cells were transfected with a mock or with a plasmid expressing PACS2 48 h before experiment and grown in normal medium. (*O*) Mitochondrial potential of rictor KO and control MEFs infected adenovirally with LacZ, Akt or Akt-S473D, measured by TMRM staining by FACS. Cells were growing in normal medium and infected 48 h before experiment. Results are shown as mean ± SEM and normalized to wild-type cells. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

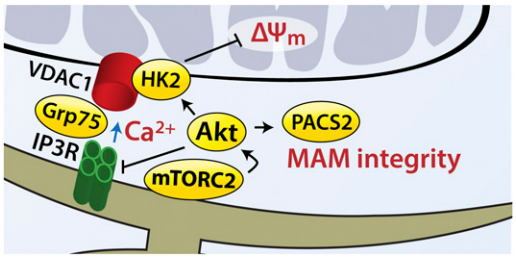

Fig. S7. Model of mTORC2-Akt signaling at MAM. mTORC2 localizes to the ER subdomain MAM. At MAM, mTORC2 can phosphorylate and active Akt. mTORC2-activated Akt can phosphorylate: (*i*) IP3R3, thereby inhibiting its potential to release calcium at MAM; (*ii*) PACS2, thereby controlling MAM integrity; (*iii*) HK2, thereby controlling mitochondrial potential and, thus, energy production.