PKG1-modified TSC2 regulates mTORC1 activity to counter adverse cardiac stress

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The mechanistic target of rapamycin complex-1 (mTORC1) coordinates regulation of growth, metabolism, protein synthesis and autophagy1. Its hyperactivation contributes to disease in numerous organs, including the heart1–3, although broad inhibition of mTORC1 risks interference with its homeostatic roles. Tuberin (TSC2) is a GTPase-activating protein and prominent intrinsic regulator of mTORC1 that acts through modulation of RHEB (Ras homologue enriched in brain). TSC2 constitutively inhibits mTORC1; however, this activity is modified by phosphorylation from multiple signalling kinases that in turn inhibits (AMPK and GSK-3β) or stimulates (AKT, ERK and RSK-1) mTORC1 activity4–9. Each kinase requires engagement of multiple serines, impeding analysis of their role in vivo. Here we show that phosphorylation or gain- or loss-of-function mutations at either of two adjacent serine residues in TSC2 (S1365 and S1366 in mice; S1364 and S1365 in humans) can bidirectionally control mTORC1 activity stimulated by growth factors or haemodynamic stress, and consequently modulate cell growth and autophagy. However, basal mTORC1 activity remains unchanged. In the heart, or in isolated cardiomyocytes or fibroblasts, protein kinase G1 (PKG1) phosphorylates these TSC2 sites. PKG1 is a primary effector of nitric oxide and natriuretic peptide signalling, and protects against heart disease10–13. Suppression of hypertrophy and stimulation of autophagy in cardiomyocytes by PKG1 requires TSC2 phosphorylation. Homozygous knock-in mice that express a phosphorylation-silencing mutation in TSC2 (TSC2(S1365A)) develop worse heart disease and have higher mortality after sustained pressure overload of the heart, owing to mTORC1 hyperactivity that cannot be rescued by PKG1 stimulation. However, cardiac disease is reduced and survival of heterozygote Tsc2S1365X knock-in mice subjected to the same stress is improved by PKG1 activation or expression of a phosphorylation-mimicking mutation (TSC2(S1365E)). Resting mTORC1 activity is not altered in either knock-in model. Therefore, TSC2 phosphorylation is both required and sufficient for PKG1-mediated cardiac protection against pressure overload. The serine residues identified here provide a genetic tool for bidirectional regulation of the amplitude of stress-stimulated mTORC1 activity.

Hearts that are subjected to sustained pressure overload develop pathological growth and show a reduction in cardiac function (Extended Data Fig. 1a). This pathology is accompanied by mTORC1 activation, which induces increased phosphorylation of p70S6K and eIF4E binding protein-1 (4E-BP1)—which stimulates the transcription and translation of genes—and Unc-51-like kinase-1 (ULK1), which reduces autophagy14 (Fig. 1a and Extended Data Fig. 1b). PKG1 activation by oral administration of the phosphodiesterase-type-5 inhibitor sildenafil suppresses these changes. In addition, PKG1 activation increases the expression of microtubule-associated protein light-chain 3-II (LC3-II) while reducing the expression of p62 (Fig. 1b and Extended Data Fig. 1c) and the aggregation of myocardial proteins (Extended Data Fig. 1d), all consistent with increased autophagy. These changes are also induced by everolimus, a relatively selective mTORC1 inhibitor. In isolated cardiomyocytes that have been stimulated using endothelin-1 (ET1), cGMP activation of PKG1 increases autophagic flux as demonstrated by an increase in the number of red puncta (autolysosomes) in cells that express a tandem fluorescent reporter (LC3-II–GFP–RFP)15 (Fig. 1c) and by a higher increase in the expression of LC3-II in bafilomycin-A1-treated cardiomyocytes (Extended Data Fig. 2a). The anti-hypertrophic effects of PKG1 are blunted by genetic silencing of autophagy related 5 (Asg5) (Extended Data Fig. 2b). Thus, PKG1 activation suppresses cardiac mTORC1 signalling, blunts growth and enhances autophagy.

To determine the mechanism by which PKG1 induces suppression of mTORC1, adult rat cardiomyocytes were exposed to cGMP for 15 min, and lysates were analysed by phosphoproteomics. Among mTORC1-complex and -regulating proteins, mass spectrometry analyses identified phosphorylation at the first of two adjacent serines (S1364 and S1365 in humans; S1365 and S1366 in mice) in a highly conserved activation domain in TSC2, upstream of the GSK-3β and AMPK phosphorylation sites (Fig. 1d and Extended Data Fig. 2c). PKG1 is among the top kinases predicted to modify either S1364 or S1365 in human TSC2 (see PhosphoNET Kinexus; http://www.phosphonet.ca/kinase-predictor.aspx?uni=P49815&ps=S1364 and http://www.phosphonet.ca/kinasepredictor.aspx?uni=P49815&ps=S1365). Phosphorylation of TSC2(S1364) in humans has been reported previously16,17. There are no reported human mutations in human TSC2(S1364); however, two children have been reported to have an S1365L mutation; each exhibited seizures but no tumours.

A commercial antibody against phosphorylated TSC2(S1365) in mice had been manufactured, but had no prior validation. Mouse embryonic fibroblasts (MEFs) show low levels of phosphorylation at baseline that increase with cGMP stimulation and are blocked by PKG1 inhibition using DT3 (Fig. 1e and Extended Data Fig. 2d). The antibody signal is present in the myocardium in vivo, increases after pressure overload and further increases after pressure overload and treatment with sildenafil but not after pressure overload and treatment with everolimus (Fig. 1f). The level of phosphorylation correlates with PKG1 activity (Fig. 1g). Analogous phosphorylation at TSC2(S1364) in humans is found in human non-failing myocardium and increases with dilated cardiomyopathy (Fig. 1h). To test antibody specificity, rat cardiomyocytes were transfected with human wild-type haemagglutinin (HA)-tagged TSC2(WT), HA-tagged TSC2(S1364A) or HA-tagged TSC2(S1364E) at matched expression levels (Extended Data Fig. 2e), and cells were stimulated with ET1 to activate PKG1 (Extended Data Fig. 3a, b) and induce TSC2 phosphorylation. The antibody signal increased only when TSC2(WT) was expressed (Fig. 1i and Extended Data Fig. 3c). Identical results were obtained when human TSC2 was mutated at the adjacent S1365 (Extended Data Fig. 3d); thus, interference with either serine prevents PKG1 phosphorylation.

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https://doi.org/10.1038/s41586-019-0895-y
PKG1 directly phosphorylates TSC2, as demonstrated by radiolabelling of TSC2 upon co-incubation of recombinant PKG1α—the primary cardiovascular isomorph—with HA–TSC2 and [γ-32P]ATP (Fig. 1j). Radiolabelling of TSC2 also occurred when human HA–TSC2(S1364A) (Fig. 1j) or human Flag–TSC2(S1365A) (Extended Data Fig. 3e) was used, indicating that additional phosphorylation sites exist. We also tested whether PKG1α (the primary cardiovascular isomorph) directly modifies TSC2 in the presence of proteins isolated from whole-cell lysates using mutated PKG1α (M438G)18—which uniquely binds to and enlarged sulfonated ATP19. This assay revealed a TSC2 thio phosphate ester (PKG1α phosphorylation) in TSC2(WT) and both S1364A and S1365A mutations (Extended Data Fig. 3f).

We next tested the functional importance of the serine residues. Rat cardiomyocytes were transfected with TSC2(WT), human HA–TSC2(S1364A) or human HA–TSC2(S1364E) and then stimulated with ET1 for 48 h (Fig. 2a, b). Basal expression of rat Nppb mRNA (a hypertrophy biomarker) was low, regardless of which TSC2 construct was overexpressed. ET1 increased Nppb expression the most in human TSC2(S1364A)-expressing cells and the least in human TSC2(S1364E)-expressing cells, each compared to TSC2(WT) (Fig. 2a). Nppb expression decreased with PKG1α activation only in TSC2(WT)-expressing cells, consistent with the lack of phosphorylation of human TSC2 at S1364 with either mutation (Fig. 1i). Very similar results were obtained when human TSC2 was mutated at S1365A or S1365E (Extended Data Fig. 4a), indicating that mutations in either serine alone are sufficient to induce a similar biological response. In comparison to expression of TSC2(WT), TSC2(S1365A) expression increased whereas TSC2(S1365E) expression attenuated mTORC1 activity after stimulation using ET1; neither mutation had an effect at baseline. LC3-II protein expression increased and p62 expression decreased after expression of TSC2(S1365E), supporting enhanced autophagy; the opposite occurred after expression of TSC2(S1365A). This was observed regardless of which serine was mutated (human TSC2(S1364); Fig. 2b and Extended Data Fig. 4b; human TSC2(S1365); Extended Data Fig. 4c, d). An increase in the number of red LC3-II punctae indicate that there is an increase in autophagic flux with expression of TSC2(S1365E) (Fig. 2c). Phenylephrine treatment mimicked the effects of ET1 (Extended Data Fig. 5a), and expression of TSC2(S1364A) depressed the modulation of PKG1 of LC3-II and p62 (Extended Data Fig. 5b). Therefore, mutants with substitutions at either of the two adjacent serine residues in TSC2 either amplify or attenuate stress-stimulated mTORC1 regulation, and the effects of PKG1 on growth and autophagy are either markedly impaired (S1365A) or mimicked (S1365E) by single point mutations in either residue.

TSC2 predominantly modulates mTORC1 by regulating RHEB–GTP binding, and silencing of Rheb blocked ET1-stimulated activation of mTORC1 in TSC2(WT)– and TSC2(S1365A)-expressing rat cardiomyocytes. TSC2(S1365E)-expressing cells exhibited low mTORC1 activity despite ET1 stimulation, regardless of whether Rheb was
silenced or not (Fig. 2d and Extended Data Fig. 5c). The serine residues are proximate to residues that are targeted by AMPK and GSK-3β, the latter of which requires AMPK co-activation of TSC2. To test whether the S1365A mutation prevents TSC2–mTORC1 control through AMPK, Tsc2 knockout MEFs that expressed TSC2(WT), TSC2(S1365A) or an empty vector were exposed to ET1 followed by treatment with 2-deoxyglucose (2-DG), which inhibits glycolysis to stimulate AMPK. Tsc2 knockout MEFs showed constitutive activation of mTORC1 that was reasonably insensitive to 2-DG. Expression of TSC2(WT) or TSC2(S1365A) reduced the activity of mTORC1, and this activity was further depressed similarly in each of these two groups by 2-DG in concordance with phosphorylation of the AMPK–phosphorylation site at S1387 (Fig. 2e–h). Thus, a silencing or phosphomimetic TSC2 mutation at human S1364 or S1365 does not impede energy-responsive inhibition of mTORC1 associated with AMPK-phosphorylation at neighboring TSC2 sites.

Mice with a global Tsc2(S1365A) knock-in mutation were generated using CRISPR–Cas9 gene editing (Fig. 3a and Extended Data Fig. 6a) to assess the role of TSC2 in vivo. Mice were born at normal Mendelian ratios, grew and developed normally, and had similar cardiac structure and function compared to their littermate controls (Extended Data Table 1).

In addition, the expression levels of myocardial TSC2 were similar to those in littermate controls (Extended Data Fig. 6b). Heterozygote (Tsc2S1365A/WT), homozygote (Tsc2S1365A/S1365A) and control (Tsc2WT/WT) mice were subjected to pressure overload, and randomized to either treatment with vehicle or sildenafil. Mortality after pressure overload in vehicle-treated mice was markedly higher in Tsc2S1365A/WT and Tsc2S1365A/S1365A mice compared to Tsc2WT/WT mice. Notably, sildenafil prevented the increase in mortality in Tsc2S1365A/WT but not Tsc2S1365A/S1365A mice (Fig. 3b). Pressure-overload-induced hypertrophy and dysfunction of the heart and increased lung weight were more pronounced in Tsc2S1365A/WT and Tsc2S1365A/S1365A mice compared to Tsc2WT/WT mice, and sildenafil prevented the increase in mortality in Tsc2S1365A/WT but not Tsc2S1365A/S1365A mice (Fig. 3c, d and Extended Data Fig. 6c). Activity of mTORC1 and aggregation of myocardial proteins were greater and autophagy (increased p62 expression and decreased LC3-II expression) was depressed following pressure overload in Tsc2S1365A/WT and Tsc2S1365A/S1365A mice compared to Tsc2WT/WT mice, and this reduction in autophagy was also reversed by sildenafil in the hearts of Tsc2S1365A/WT and Tsc2WT/WT mice, but not Tsc2S1365A/S1365A mice (Fig. 3e, f and Extended Data Fig. 6d, e). These findings were similar in both male and female mice. Activation of mTOR complex-2
was unaltered by the TSC2(S1365A) mutation (Extended Data Fig. 7a). Tsc2(S1365A/WT) mice randomized to everolimus treatment (compared to vehicle, each starting three days before pressure overload) showed similar levels of mortality (Fig. 4a) and heart disease (Fig. 4b, c) after pressure overload as wild-type controls, and had increased levels of autophagy (Extended Data Fig. 7b), supporting a role for mTORC1 hyperactivation, which is induced by expression of TSC2(S1365A). Together, these results show that TSC2(S1365A) in mice acts as an autosomal dominant mutation that amplifies stress-stimulated, but not basal, mTORC1 activity in vivo. In addition, we show that pS1365 is required for PKG1-mediated mTORC1 suppression and amelioration of heart disease induced by pressure overload.

Tsc2(S1365E) knock-in mice were also generated (Extended Data Fig. 8a) to test whether a phosphorylation-mimicking mutation confers effects that are opposite to the S1365A mutation. Mice were also born at normal Mendelian ratios, and had normal cardiac morphology and function (Extended Data Table 1). In contrast to Tsc2(S1365A/WT) and Tsc2(S1365E/WT) mice, both Tsc2(S1365E/S1365E) and Tsc2(S1365A/S1365A) mice were markedly protected against pressure overload, and showed minimal hypertrophy and better heart function compared to Tsc2(WT/WT) controls (Fig. 4d, e). Baseline mTORC1 activity after expression of TSC2(S1365E) is similar to TSC2(WT), but remains low after pressure overload, whereas expression of LC3II increases and p62 decreases (Fig. 4f and Extended Data Fig. 8b). In vivo autophagic flux is higher in Tsc2(S1365E/S1365E) mice and lower in Tsc2(S1365A/S1365A) compared to wild-type mice (Extended Data Fig. 8c). Both Tsc2(S1365E/S1365E) and Tsc2(S1365A/S1365A) mice also have reduced aggregation of myocardial proteins (Fig. 4g).
and increase in heart and lung weight normalized to tibial length induced by pressure overload and pressure overload treated with everolimus. The number of mice per group is shown.

The increase in heart and lung weight normalized to tibial length induced by pressure overload is reversed by everolimus treatment in Tsc2(S1365A) mice; data are mean±s.d.; Kruskal–Wallis test with Dunn’s multiple comparisons test versus pressure overload alone: ***P = 0.01 versus sham and ****P = 0.001 versus pressure overload and everolimus; *P = 0.021 versus sham and ***P = 0.0005 versus pressure overload and everolimus.

Echocardiograms of Tsc2 WT/WT and Tsc2(S1365E/S1365E) mice subjected to pressure overload, sildenafil failed to alter expression of LC3-II and p62 or aggregation of myocardial proteins, supporting a key role of PKG1–TSC2 modulation at mouse TSC2(S1365). Whether genetically preventing autophagy abrogates PKG1 amelioration of heart disease in vivo, and if so whether it requires human TSC2(S1364) or TSC2(S1365), remains to be tested.

Our results identify PKG1 as a direct mediator of post-translational regulation of TSC2, and PKG1(T158C) (numbering based on the human sequence), the fact that PKG1 does not phosphorylate TSC2 regardless of which residue is mutated, and that cell behaviour after mutations of either serine 1364 or 1365 (based on the human sequence) remains to be tested.
that pathological stress stimulates TSC2 phosphorylation in cardiomyocytes and the myocardium along with activation of mTORC1, whereas preventing this phosphorylation by substituting alanine for the PKG1-modified serines amplifies the pathology, supports a role for the phosphorylation of TSC2 at this site as negative feedback on mTORC1 activity. The protective effects induced by substituting serine with glutamic acid or the effects of more selective phosphorylation by PKG1 support this idea further. Mammalian cells ubiquitously express TSC2, mTOR and related mTORC1–complex proteins, and many cell types also express PKG1. Therefore, our data have implications beyond the heart, including to potential therapeutic roles for PKG1 activators in diseases in which altered modulation of mTORC1 is present and/or desired. Furthermore, genetic modulation of the identified phosphorylation sites provides a method of mTORC1 control with potential use in adoptive cell therapies, by providing a more nuanced and flexible method of mTORC1 regulation.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41586-019-0899-y.

Received: 4 November 2018; Accepted: 13 December 2018; Published online 30 January 2019.

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METHODS
Data reporting. While no statistical methods were used to predetermine sample size for each assay, we based the group size whenever possible on prior experience with similar assays and variance. The surgeon who performed pressure overload in the mice, and sonographer who obtained and interpreted the echocardiographic images were fully blinded to genotype and/or treatment. All tissues were isolated by an independent investigator with no knowledge of the genotype. Subsequent molecular and biochemical studies were performed with knowledge of the genotype from which the extracts had been obtained.

Animal models. Mice expressing global TSC2 knock-in mutations MmtTSC2(S1365A) or MmtTSC2(S1365E) (Fig. 3a and Extended Data Figs. 6a, 8a) were newly generated using CRISPR-Cas9 gene editing (Transgenic Core Laboratory, Johns Hopkins University). Tsc2 guide RNA was designed using an algorithm developed by V. Ranganathan and cloned into pSpCas9(BB)-2A-Puro (PX459) V2.0 (a gift from F. Zhang, Addgene plasmid 62988) and pUC57-sgRNA (a gift from X. Huang, Addgene plasmid 51352) expression vectors. DNA cleavage was tested in mouse N2a cells using the Surveyor Mutation Detection kit (Integrated DNA Technologies) according to the previously published protocol10. In vitro transcription was performed for both Cas9 (from a modified pX330-U6-Chimeric_BB-CBh-hSpCas9 plasmid, originally a gift from F. Zhang, Addgene plasmid 42230) and guide RNA using the Ambion mMESSAGE mMACHINE kit and NEB HiScribe T7 High Yield RNA Synthesis kit, respectively. Single-sequence oligodeoxynucleotide (ssODN) for the S1365A or S1365E point mutations were purchased from Integrated DNA Technologies. C57BL/6j blastocyst injections were performed by members of the Johns Hopkins Transgenic Mouse Core Laboratory with a minimum of 12.5 ng guide RNA and 25 ng gRNA ssODN. We received five founder mice that were positive for the Tsc2<sup>S1365(S)</sup> transgene and three founder mice that were positive for the Tsc2<sup>S1365E</sup> transgene. Both colonies of mice were backcrossed with mice on a C57BL/6J background, generating mice with a 98.5% (S1365E) and 99.3% (S1365A) purity. The genetic backgrounds of mice were assessed at DartMuse Speed Congenic Core Facility at the Geisel School of Medicine at Dartmouth. DartMuse uses the Illumina Infinium Genotyping Assay to analyze a custom panel of 5,307 single-nucleotide polymorphisms (SNPs) spread throughout the genome. The raw SNP data were analyzed using DartMouse’s SNAP-Map and Map-Synth software, allowing the determination for each mouse of the genetic background at each SNP location. We maintained each colony with the breeding strategy of a heterozygous male and a heterozygous female, yielding knock-in homozygous mice, knock-in heterozygous mice, and littermate wild-type mice at a 25:50:25 ratio.

Pressure-overload model. Pressure overload was induced by trans-aortic constriction as previously described15. Sham controls underwent similar surgery without ligature constriction. Age-matched (2–3 months) and weight-matched (generally, 25–30 g) littermates were randomly allocated into pressure overload or sham-surgery groups. Female and female-sham groups were used in all experiments. Mice were followed for up to 6 weeks after pressure overload, in some instances, co-treated with everolimus (Sigma; oral gavage, 10 mg kg<sup>−1</sup> day<sup>−1</sup>) or sildenafil (Pfizer or Wako Pure Chemical Industries, 200 mg kg<sup>−1</sup> day<sup>−1</sup> mixed in soft diet, Bioserv) or appropriate matched vehicle. Drug treatment started either 1 week following pressure overload, or several days before pressure overload. All of the protocols were approved by the Johns Hopkins Medical Institutions Animal Care and Use Committee. The studies were in compliance with all ethical regulations.

Conscious mouse echocardiography. Intact heart morphology and function were measured in conscious mice by serial M-mode transthoracic echocardiography (VisualSonics Vevo 2100, 18–38 MHz transducer; FUJIFILM VisualSonics). Images were obtained and analysed using VisualSonics image software, by an individual blinded to the conditions of the animal.

TSC2 expression plasmids and adenosine. An N-terminally Flag-tagged human TSC2 expression plasmid was provided by B. Manning (Harvard University) and is available from Addgene (Plasmid 14129). Based on this, we further generated five new constructs, one in which Flag was replaced by an HA sequence (also at the N terminus), and then each wild-type vector was further modified to generate human HA-TSC2(S1364A), HA-TSC2(S1364E), Flag-TSC2(S1365A) and Flag-TSC2(S1365E). Adenosine was also developed expressing the Flag-tagged wild-type sequence or human TSC2(S1365A) or TSC2(S1365E) sequences (Weglen) and used for studies involving Tsc2 knockout and Tsc2 wild-type MEFs. These cell lines were provided by B. Manning as first generated by D. Kwiatkowski37.

Neonatal rat cardiomyocyte studies. Neonatal rat cardiomyocytes (NRCMs) were isolated and cultured at 1 million cells per well in six-well plates for 24 h in DMEM with 10% FBS and antibiotics before study, as previously described18. Hypertrophy was stimulated with ET1 (10 nM, Sigma), phenylephrine (100 μM, Sigma) or both, for 24 h in serum-free DMEM supplemented with 0.1% insulin–transferrin–selenium (Life Technologies). Studies were also performed in NRCMs first transfected with plasmids expressing TSC2(WT), TSC2(S1365A) or TSC2(S1365E) (human S1364 or human S1365) (5 μg per well), using Takara Clontech XFeCT as per the manufacturer’s protocol. Cells were provided 24 h before exposure to stimulation with ET1 or vehicle in combination with relevant other structures as described in the study.

Tsc2 knockout MEFs. MEFs were cultured in DMEM supplemented with 10% FBS and 1% antibiotics until 50–60% confluence, at which time the cells were transfected with Tsc2 constructs or empty vector, or infected with adenosine expressing TSC2(WT), TSC2(S1365A) or TSC2(S1365E). Expression vectors were used a CMV promoter. After an additional 24 h of culturing, transfected MEFs were treated with 8-bromo-cGMP (100 μM), D3 (1 μM), 2-DG (100 μM) or vehicle control.

Human myocardium analysis. Human myocardium was obtained in accordance with institutional review board approvals at Johns Hopkins University and the University of Pennsylvania. The use of human subject material was in compliance with all ethical regulations. Failing human hearts were obtained at the time of explant surgery, and non-failing controls at time of the collection of other organs. Left ventricular free wall tissue was collected at the University of Pennsylvania in ice-cold cardioplegia and rapidly frozen in liquid nitrogen. Informed consent was obtained from failing heart human tissue donors. The family or legal representative provided consent for organ collection from deceased donor controls. For the non-failing controls, the age was 52.8 ± 15.4, 6 males, 6 females, no clinical history of heart failure; for the heart failure group, the mean age was 51.3 ± 12.1, 8 males, 4 females (P = not significant for sex distribution between groups), and all were patients with non-ischaemic dilated cardiomyopathy with severe left ventricular dysfunction who then underwent cardiac transplantation.

Immunoblot analysis. Whole-cell lysates was extracted (Cell Signaling Technology, 9803) and RNA was isolated by direct lysis or using TRIzol Reagent (Invitrogen). Gene expression by quantitative RT–PCR. Total RNA isolated from left ventricular myocardium or cultured NRCMs (TRIzol Reagent, Invitrogen), was reverse transcribed to cDNA (High Capacity RNA-to-cDNA Kit, Applied Biosystems) and underwent PCR amplification using TaqMan probes for atrial natriuretic peptide (Nppa) (mouse Mm01255747_g1, rat Rn00664637_g1), B-type natriuretic peptide (Nppb) (mouse Mm01255770_g1, rat Rn00586041_m1), regulator of calcium (Rcan1) (mouse Mm01213406_m1, rat Mm01484942_m1), tuberous sclerosis complex 2 (Tsc2) (mouse Mm00442004_m1, rat Rn00562086_m1), glyceraldehyde-3-phosphate dehydrogenase (Gapdh) (mouse Mm00801030_m1, rat Rn01757613_g1) (Applied Biosystems). The threshold cycle value was determined using the crossing point method and samples were normalized to Gapdh for each run. Bafilomycin A1 autophagy-flux assay. For in vitro studies, NRCMs were cultured as described above, stimulated with cGMP (50 μM) for 15 min, and then treated with either vehicle or bafilomycin A1 (BFA, 1 μM) (Sigma) for 3 h. For in vivo studies, Tsc2<sup>Wt/Wt</sup>, Tsc2<sup>S1365A/S1365A</sup> and Tsc2<sup>S1365E/S1365E</sup> mice received two intraperitoneal injections of BFA (3 μM kg<sup>−1</sup> day<sup>−1</sup>) 90 min apart. The myocardium was collected 90 min after the second injection, and protein extracts were analysed by immunoblot for expression of LC3-II. The relative increase in expression with versus without BFA indexes autophagic flux.

Protein aggregation assays. Protein aggregation was measured using ProtoArray<sup>®</sup> HD (Invitrogen Life Technologies) as per manufacturer’s instructions. Ventricular myocardial lysates (Cell Signaling lysis buffer) was obtained, protein concentration assayed and 10 μg protein loaded into a 96-well microplate and protein aggregates were analysed using the Proteostat assay kit (Enzo Life Sciences) following the
manufacturer's instructions. After background subtraction, values were normalized to wild-type sham.

For a second assay, 1 mmol l⁻¹ phenylmethanesulfonyl fluoride and a phoshSTOP tablet (Sigma-Aldrich/Roche) were added to myocardial lysates. This was centrifuged at 8,000g for 4° C for 10 min, the supernatant (soluble fraction) was extracted and the pellet was resuspended in insoluble extraction buffer (40 mmol l⁻¹ Tris HCl (pH 8.8), 1% SDS and 8% glycerol), boiled for 5 min and recentrifuged at 3,000g for 5 min. Then, 2.5 μg of the supernatant (insoluble fraction) was fractioned through a nitrocellulose membrane (pore diameter, 0.22 μm, Millipore) using a dot-blot apparatus (BioRad) and immunoprobed for ubiquitin and α-tubulin.

Tandem fluorescent LC3-II autophagic flux assay. NRMCs were infected with an adenovirus (10 MOI) expressing a tandem fluorescent (GFP–RFP) tagged LC3-II. This expresses LC3-II with both green and red fluorescent tags as the autophagosomal membrane is forming; but upon merging with the acidic lysosome (autolysosome), the GFP signal is quenched, leaving RFP. The increase in RFP provides a marker of autophagic flux. In some studies, cardiomyocytes were further transfected with a plasmid encoding TSC2(WT), TSC2(S1365A) or TSC2(S1365E) and stimulated for 48 h with ET1 (10 nM). Dot counts for both colours per cell were determined using ImageJ software (version 1.52a, NIH).

In vitro PKG1 activity. PKG1 activity was assessed by in vitro colorimetric assay (Cycldex, CY-1161, MBL International) following the manufacturer's instructions. The assay provides CGMP substrate and a kinase-specific peptide target to assess phosphorylation activity.

Proteomic analysis of PKG1 phosphokinome. Freshly isolated adult cardiomyocytes were obtained from male Wistar rats as previously described 26, and divided into two aliquots, each relaxed in Tyrode buffer (140 mmol NaCl, 5 mmol KCl, 10 mmol HEpes, 1 mmol glucose, 1 mmol MgCl₂, 1 mmol CaCl₂, pH 7.45). Cells were then exposed to 1 mmol l⁻¹ 8-bromo-cGMP or Tyrode solution for 10 min to stimulate intracellular PKG1 activity. Cells were then centrifuged for 1 min at 1,000g, the supernatant was removed and the pellet frozen in liquid nitrogen and stored at −80 °C. Frozen samples (n = 3 per group) were then lysed in an 8 M urea and 0.5% SDS solution with brief sonication, and the protein concentration was determined by the BCA method. For each sample, 200 μg of total protein was digested with trypsin/Eys-C protease mixture (Promega), samples were desalted on 10 mg Oasis HLB cartridges (Waters) and eluted in 300 μl of 80% acetonitrile (ACN). 5% trihydroacetic acid, 1 M glycolic acid and enriched by titanium dioxide (TiO₂). Enriched peptides were desalted as above but eluted in 200 μl of 80% ACN, 0.1% formic acid and dried under vacuum. Dried peptides were re-suspended in 20 μl of 0.1% formic acid for LC–MS/MS analysis. Samples (4 μl) were injected in duplicate onto an EASY-nLC 1000 (mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in ACN) connected to a Q-Exactive Plus (Thermo Scientific) equipped with a nano-electrospray ion source. Raw MS/MS data was searched using the Sorcerer 2TM-SEQUEST algorithm (Sage-N Research) using default peak extraction parameters. Post-search analysis was performed using Scaffold 4 (Proteome Software) with protein and peptide probability thresholds set using default peak extraction parameters. Post-search analysis was performed using Graphpad Prism software v.8 (2017).

Statistical analysis. Data annotations (for example, mean ± s.d. or mean ± s.e.m.), the number of experiments and biological independent samples, analyses—including multiple comparisons tests—and absolute P values for comparisons are provided for each figure in the figure or the legend. The majority of summary data are presented as mean ± s.d., analysed using one-way or two-way ANOVAs and using Tukey or Sidak multiple pairwise comparisons tests. Statistical analyses were performed using Graphpad Prism software v.8 (2017).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability

The authors declare that the data supporting the findings of this study are available within the paper and the Supplementary Information. Numerical values corresponding to figures that describe the results from in vivo model studies are provided as separate Source Data for Figs. 1f–h, 2a, 3d, 4c, e and Extended Data Fig. 1a. Other source data related to the study are available from the corresponding author upon reasonable request. Any reagents developed for this study, including novel plasmids, viral vectors and the Tsc2 knock-in mouse models can be made available on direct request to the corresponding author.

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Extended Data Fig. 1 | See next page for caption.
Both mTORC1 inhibition (everolimus) and PKG1 activation (sildenafil) prevent pathological heart growth, dysfunction, hypertrophic gene expression, mTORC1 activation and myocardial protein aggregation. a. Heart weight/tibial length, lung weight/tibial length, cardiac ejection fraction, mRNA expression of *Nppa*, *Nppb* and *Rcan1* (encoding A- and B-type natriuretic peptide and regulator of calcineurin 1, respectively, and each normalized to *Gapdh*) from the same study that generated the data shown in Fig. 1a, b. Mice were subjected to 6 weeks of pressure overload (PO) from trans-aortic constriction or to sham surgery, and pressure-overloaded mice were further treated with vehicle, sildenafil (200 mg kg\(^{-1}\) day\(^{-1}\)) or everolimus (10 mg kg\(^{-1}\) day\(^{-1}\)) starting 1 week after pressure-overload surgery. Data are mean ± s.e.m.; \(n = 6\) biologically independent experiments, one-way ANOVA with Tukey multiple comparisons test, \(^{\dagger}P \leq 6 \times 10^{-5}\) versus the other three groups, \(^{\ddagger}P = 1 \times 10^{-6}\) versus sham, \(^{\star}P = 0.007\) versus pressure overload and sildenafil, \(P = 0.002\) versus pressure overload and everolimus; \(^{\ddagger\ddagger}P = 0.02, \ ^{\dagger\dagger}P < 0.007, \ ^{*}P = 0.02\) versus Sham; \(^{\dagger}P \leq 0.0005\) versus all other groups. b, c. Summary analysis for immunoblots displayed in Fig. 1a, b. Data are mean ± s.e.m.; \(n = 6\) biologically independent experiments as in a. One-way ANOVA with Tukey multiple comparisons test, \(^{\dagger\dagger\dagger}P < 1 \times 10^{-6}, \ ^{\ddagger\ddagger}P < 1 \times 10^{-5}\) versus the other three groups, \(^{\dagger}P < 1 \times 10^{-6}\) versus sham, \(P = 0.0003\) versus pressure overload and vehicle, \(^{*}P = 0.002\) versus sham. d. Filter trap assay from myocardium obtained from same mouse experiment, with membranes probed for ubiquitin and \(\alpha\)-tubulin. \(n = 4\) biologically independent experiments, mean ± s.e.m., one-way ANOVA with Tukey multiple comparisons test, \(^{*}P = 0.002, \ ^{\ddagger}P = 0.001\) versus sham.
Extended Data Fig. 2 | See next page for caption.
**Extended Data Fig. 2** | PKG1 activation enhances autophagic flux and this is required for anti-hypertrophic efficacy and leads to TSC2 phosphorylation at S1364 (human; 1365 mouse; 1366 rat).

**a,** Immunoblot of LC3-II in NRCMs with and without BFA treatment to block autophagy by preventing lysosomal proteolysis. The relative increase in LC3-II expression without versus with BFA treatment indexes autophagic flux. An example blot is shown on the left, summary data on the right. \( n = 4 \) biologically independent experiments; data are mean ± s.d., two-way ANOVA with Tukey multiple pairwise comparisons test, \( P < 0.0001 \) for interaction between ±BFA and drug treatment; \( ^*P = 1.5 \times 10^{-5}, ^{†}P < 1 \times 10^{-6}, ^{‡}P = 0.013, ^{‡}P = 0.0002 \) for within group comparison ±BFA; \( ^{‡}P < 1 \times 10^{-4} \) versus each of the other groups with BFA. **b,** Effect of siRNA gene knockdown of Atg5 (siATG5) versus scrambled control (Scr-siRNA) on Nppb gene expression in NRCMs treated with ET1 with or without sildenafil. \( n = 6 \) biologically independent samples, data are mean ± s.d., one-way ANOVA with post hoc Tukey test: \( ^{†}P = 0.00004 \) versus vehicle, \( ^{‡}P = 0.0004 \) versus cGMP. **c,** Mass spectrometry identifies Tsc2 S1366 in rat (equivalent to human S1364 and mouse S1365) as a phosphorylation target of PKG1. Adult rat ventricular myocytes were exposed to cGMP to stimulate PKG1 activity and mass spectrometry was performed on three independent replicates. **d,** Summary of immunoblot experiment in Fig. 1e. MEFs treated with 8-bromo-cGMP with and without the PKG1 inhibitor DT3. \( n = 6 \) biologically independent samples, data are mean ± s.d., one-way ANOVA with post hoc Tukey test: \( ^{†}P = 0.00004 \) versus vehicle, \( ^{‡}P = 0.0004 \) versus cGMP. **e,** Immunoblot for TSC2 (antibody that recognizes the C terminus) in cardiomyocytes expressing native protein, or transduced with TSC2(WT), TSC2(S1365A) or TSC2(S1365E) using plasmid vector transfection. Expression levels were similar with each plasmid at twice the level of non-transduced cells. \( n = 4 \) biologically independent samples, data are mean ± s.d., one-way ANOVA with post hoc Tukey test, \( ^*P = 0.0004 \) versus control.
Extended Data Fig. 3 | PKG1 is activated by ET1 in rat cardiomyocytes and results in TSC2 phosphorylation detected by a human TSC2(S1364) antibody in cells expressing human TSC2(WT) but not human TSC2(S1364A) or TSC2(S1365A); this phosphorylation occurs by direct modification of TSC2 by PKG1α. a, PKG1 activation in cardiomyocytes expressing human TSC2(WT), TSC2(S1365A) or TSC2(S1365E) and stimulated with ET1 (10 nM) versus vehicle for 48 h. Data are mean ± s.d., n = 18 biologically independent samples, two-tailed unpaired Student’s t-test. b, From same experiment, PKG1 activation is found to be independent of the form of TSC2 expressed; n = 6 biologically independent samples, box and whisker and raw data plots. Data normalized to median for wild type (without ET1); P = 0.0004 for ET1 effect, P > 0.8 for TSC2 genotype effect by two-way ANOVA. c, Summary data for phosphorylated/total human TSC2(S1364) from the rat cardiomyocyte ET1-stimulation experiment shown in Fig. 1f. Data are mean ± s.d., n = 6 biologically independent samples from three experiments, one-way ANOVA with Tukey multiple comparisons test, #P = 0.003 versus TSC2(S1365E), P = 0.0003 versus TSC2(S1365A). d, Antibody raised against TSC2(S1365) (mouse) (equivalent to human TSC2(S1364)) shows increased TSC2 phosphorylation in rat cardiomyocytes transfected with human TSC2(WT), but not cells expressing human TSC2(S1365A) or human TSC2(S1365E). Summary from three independent replicates yielding n = 6 biologically independent samples, data are mean ± s.d., one-way ANOVA with Tukey multiple comparisons test, *P = 0.0002 versus TSC2(S1365E), P = 0.0007 versus TSC2(S1365A). The results are identical to those using mouse TSC2(S1365) (human TSC2(S1364)) mutants displayed in Fig. 1i, indicating that mutations at either serine (human sequence: S1364 or S1365; mouse sequence S1365 or 1366) prevents phosphorylation of the other and/or its detection by the phosphorylation-specific antibody. e, Direct TSC2 phosphorylation by recombinant PKG1α detected by autoradiography on human Flag–TSC2(WT) and Flag–TSC2(S1365A). Experiments were replicated three times (n = 6 biologically independent samples) with identical results. The result is identical to that in Fig. 1j with human HA–TSC2(S1364A). f, Direct TSC2 phosphorylation by PKG1α in lysates from TSC2 knockout HEK293 cells expressing human TSC2(WT) or human HA–TSC2(S1364A) or human Flag–TSC2(S1365A), PKG1α(M438G), and N6-benzyl-ATP-γ-S, and probed for thiophosphate ester. Top, data with human TSC2(S1364) mutated; bottom, with human TSC2(S1365) mutated. The results are identical. n = 6 biologically independent samples for each assay.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Human TSC2(S1364) or TSC2(S1365) mutated to glutamic acid (S1364E or S1365E) suppresses ET1-stimulated cardiomyocyte hypertrophy and mTORC1 activation, whereas mutation to alanine (S1364A or S1365A) amplifies both. a, Nppb mRNA expression (pathological hypertrophy gene marker) in rat cardiomyocytes transfected with human Flag–TSC2(WT), Flag–TSC2(S1365A) or Flag–TSC2(S1365E) and then exposed to 48 h ET1 (to induce hypertrophy) or to vehicle. Activation of PKG1 by sildenafil reduces ET1-stimulated Nppb in TSC2(WT)-expressing cells, but not in cells expressing TSC2(S1365A) or TSC2(S1365E) mutants. TSC2(S1365E) expression depresses the increase in Nppb expression with ET1 stimulation, whereas TSC2(S1365A) expression enhances it. These results are nearly identical to those shown in Fig. 2a in which the human TSC2(S1364) (first serine of the duplet) was mutated. This shows that genetic modulation of either serine results in the same biological modulation of ET1 stimulation on growth and mTORC1 activity. Data are mean ± s.d., n = 6 biologically independent experiments, one-way ANOVA with Tukey multiple comparisons test, *P < 1 × 10^{-6} versus other TSC2(WT) groups, 1P = 0.002, 11P = 0.001 versus TSC2(S1365E), 2P < 1 × 10^{-6} versus TSC2(S1365E) and TSC2(S1365A) and ET1, 3P < 1 × 10^{-6} versus TSC2(S1365E) and TSC2(S1365A) and ET1, 4P < 1 × 10^{-6} versus TSC2(S1365E) and TSC2(WT) + ET1 and sildenafil. b, Summary analysis of immunoblots displayed in Fig. 1b. Values are normalized to TSC2(WT) treated with vehicle; data are mean ± s.d., n = 4 (LC3-II) or 6 (others) biologically independent experiments; one-way ANOVA with Tukey multiple comparisons test, *P ≤ 7 × 10^{-6} versus vehicle control, 1P < 1 × 10^{-6} versus TSC2(S1364A) and ET1, 2P < 5 × 10^{-6}, 3P = 0.01 versus TSC2(WT) and ET1. c, Example immunoblots from the same experiment as in a, showing changes in mTORC1 signalling proteins, p62 and LC3-II. ET1 stimulates phosphorylation of mTORC1 targets (p70S6K, 4E-BP1 and ULK1) and increases LC3-II and p62—consistent with mTORC1 activation and enhanced autophagy. Human TSC2(S1365E) reduces mTORC1 activation and p62 and increases LC3-II, whereas human TSC2(S1365A) does the opposite. This is identical to responses found using human S1364A and S1364E mutants (b and Fig. 2b), confirming the functional equivalency of either serine modification. Experiments were replicated 2–4 times, n = 4–8 biologically independent samples. d, Summary data for this experiment. Values normalized to TSC2(WT) treated with vehicle; data are mean ± s.d., n = 8 independent replicates for p70S6K and 4E-BP1, n = 6 for ULK1 and n = 4 for p62 and LC3-II. One-way ANOVA with Tukey multiple comparisons test. Results of pairwise comparisons: *P < 1 × 10^{-6} versus corresponding TSC2 genotype and vehicle, 1P ≤ 1 × 10^{-6} versus TSC2(WT) and ET1 and TSC2(S1365A) and ET1, 2P = 0.003, 3P = 0.0001, 4P = 0.06, 5P = 5 × 10^{-6}, 6P < 1 × 10^{-6} versus TSC2(WT) and ET1.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Effects of TSC2(S1365A) and TSC2(S1365E) on mTORC1 activation in response to phenylephrine; PKG1 activation of autophagy requires in part its phosphorylation of TSC2; and amplification of mTORC1 stimulation in cells expressing S1365A TSC2 mutation requires RHEB. a, NRVMs expressing TSC2(WT), TSC2(S1365A) or TSC2(S1365E) (human TSC2(S1365) was modified) and exposed to vehicle or phenylephrine (PE, 100 mM) for 48 h. Left, example immunoblot for phospho-p70S6K and total protein. Right, summary data, normalized to TSC2(WT) without phenylephrine. Data are mean ± s.d., n = 6 biologically independent samples, Kruskal–Wallis Test with Dunn's multiple comparisons test, *P = 0.003 versus corresponding vehicle; †P = 0.0013 versus TSC2(S1365E) and ET1. b, Top, example immunoblots for LC3-II and p62 in lysates obtained from Tsc2 knockout MEFs transfected with either human TSC2(WT) or TSC2(S1364A) plasmids and then treated with ET1 (10 nM) with or without cGMP (50 μM), n = 3 biologically independent samples. Bottom, summary data, normalized to TSC2(WT) without cGMP and without ET1; data are mean ± s.d., one-way ANOVA with Tukey multiple comparisons test, *P = 0.01 versus TSC2(WT) without ET1 without cGMP, P = 0.02 versus TSC2(WT) and ET1 with cGMP, †P < 1 × 10⁻⁶ versus TSC2(S1364A) without ET1 with cGMP and versus ET1 with cGMP, ‡P = 2 × 10⁻⁶ versus TSC2(WT) with ET1, †P = 5 × 10⁻⁵ versus TSC2(WT) and ET1 with cGMP, ††P = 1 × 10⁻⁶ versus TSC2(WT) without ET1 without cGMP and versus ET1 with cGMP, ###P < 1 × 10⁻⁶ versus TSC2(WT) without ET1 without cGMP and versus ET1 with cGMP. c, Summary results for Fig. 2d. Rat cardiomyocytes transfected with RHEB or scrambled (Scr) siRNA, transfected with TSC2(WT), TSC2(S1365E) or TSC2(S1365A) plasmids (human TSC2(S1365) modified) and stimulated with ET1 for 48 h. Effect of gene silencing of Rheb on RHEB protein expression (right) and on phosphorylated/total p70S6K protein expression (left). n = 4 biologically independent experiments, data are mean ± s.d., values normalized to TSC2(WT) treated with vehicle and scramble siRNA. Each plot was analysed by one-way ANOVA with pairwise Tukey multiple comparisons test, *P = 1 × 10⁻⁶ versus scramble siRNA and vehicle for TSC2(WT) and TSC2(S1365A), †P = 0.00002 versus scramble siRNA and vehicle for TSC2(WT) or TSC2(S1365A).
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | Tsc2<sup>S1365A/S1365A</sup> knock-in mouse genotyping; expression of TSC2 with or without pressure overload; and effect on pressure-overload-stimulated hypertrophy, autophagy and mTORC1 activation. a, Mouse Tsc2<sup>S1365A/S1365A</sup> knock-in genotyping by PCR detects a unique sequence based on the mutated residue as a 206-base pair (BP) fragment. This signature was used for genotyping. b, Immunoblot of TSC2 protein from Tsc2<sup>S1365A/S1365A</sup> and Tsc2<sup>WT/WT</sup> (littermate controls) for sham and pressure-overload-treated groups. There is no difference in expression levels among these groups or conditions. Experiments were repeated independently three times with identical results. c, Nppa mRNA expression normalized to Gapdh mRNA expression in Tsc2<sup>WT/WT</sup> versus Tsc2<sup>S1365A/S1365A</sup> myocardium before and after chronic pressure overload. n = 8 biologically independent experiments, data are mean ± s.d., one-way ANOVA with Tukey multiple comparisons test, *P < 1 × 10⁻⁶ versus respective sham and versus pressure overload in other two groups, †P < 1 × 10⁻⁶ versus respective sham and versus Tsc2<sup>S1365A/S1365A</sup> pressure overload and sildenafil, P = 0.0013 versus Tsc2<sup>S1365A/S1365A</sup> pressure overload and sildenafil, ‡P < 1 × 10⁻⁶ versus respective sham and Tsc2<sup>S1365A/S1365A</sup> pressure overload and sildenafil. e, Summary data for immunoblots displayed in Fig. 3e, f. n = 4 biologically independent experiments, data are mean ± s.e.m., data normalized to mean of Tsc2<sup>WT/WT</sup> sham; one-way ANOVA with Tukey multiple comparisons test, *P < 1 × 10⁻⁶ versus respective sham and pressure overload with sildenafil treatment; †P = 0.0002 versus sham, ‡P < 1 × 10⁻⁶ versus respective sham and Tsc2<sup>WT/WT</sup> and pressure overload, †P < 1 × 10⁻⁶ versus Tsc2<sup>WT/WT</sup> and pressure overload with sildenafil treatment.
Extended Data Fig. 7 See next page for caption.
Extended Data Fig. 7 | Tsc2\textsuperscript{S1365A/S1365A} knock-in mice display significantly increased mTORC1 but no change in mTORC2 activation; depressed autophagy in Tsc2\textsuperscript{S1365A/S1365A} mice subjected to pressure overload is reversed by mTOR inhibition with everolimus.

a. Immunoblots and summary quantification for mTORC2 targets from Tsc2\textsuperscript{WT/WT} and Tsc2\textsuperscript{S1365A/S1365A} mice subjected to sham or pressure-overload surgeries and treated with sildenafil or vehicle. \( n = 4 \) biologically independent experiments, box and whisker plots (median) with individual data are shown; data normalized to median of Tsc2\textsuperscript{WT/WT} sham. One-way ANOVA with Tukey multiple comparisons test. \( P \approx 0.62 \) between conditions within genotype) in the expression of mTORC2 substrates: phosphorylated (S473)/total AKT, phosphorylated (T24/T32)/total FOXO1/3 and phosphorylated(T346)/total NRDG1. *P = 0.002 versus sham, †P = 0.04 versus pressure overload, ‡P = 0.03 versus sham. However, there were no significant changes (\( P \geq 0.62 \) between conditions within genotype) in the expression of mTORC2 substrates: phosphorylated (S473)/total AKT, phosphorylated (T24/T32)/total FOXO1/3 and phosphorylated(T346)/total NRDG1.

b. LC3-II expression is unaltered while p62 expression increases from pressure overload in Tsc2\textsuperscript{S1365A/S1365A} myocardium, indicating suppression of autophagy. Both are reversed by treatment with the mTORC1 inhibitor everolimus. \( n = 6 \) biologically independent animal experiments, data are mean ± s.d., one-way ANOVA with Tukey multiple comparisons test, *P \leq 3 \times 10^{-5} \) versus other two groups. Data are normalized to the mean of sham control.
Extended Data Fig. 8 | See next page for caption.
Extended Data Fig. 8 | Generation of Tsc2S1365E/S1365E knock-in mice and impact on pressure-overload-induced mTORC1 activation, autophagy and autophagic flux in vivo. a, Strategy and guide RNA for CRISPR–Cas9 protocol to generate Tsc2S1365E/S1365E knock-in mice. b, Summary data for Fig. 4f. Phosphorylated/total p70S6K (n = 6 biologically independent experiments), p62/α-tubulin and LC3-II/total protein (n = 4 biologically independent experiments, data are mean ± s.d., values are normalized to Tsc2WT/WT sham), two-way ANOVA with Sidak’s multiple comparisons test, *P < 1 × 10⁻⁶ versus Tsc2WT/WT sham, P = 0.0012 versus Tsc2S1365E/WT with pressure overload, and P = 0.0001 versus Tsc2S1365E/S1365E with pressure overload, ¹P = 0.0008 versus Tsc2S1365E/WT sham, ²P = 0.001 versus Tsc2S1365E/S1365E sham; for p62, ³P ≤ 1 × 10⁻⁶ versus all other groups, for LC3-II, ⁴P = 0.018 and P = 0.0003 versus pressure overload Tsc2S1365E/WT and pressure overload Tsc2S1365E/S1365E, respectively, **P = 0.02 versus pressure overload Tsc2S1365E/S1365E and P = 0.0002 versus sham, ²²P < 1 × 10⁻⁶ versus sham. c, Example immunoblot and summary results for Tsc2WT/WT, Tsc2S1365E/WT and Tsc2S1365A/S1365A mice treated with BFA or vehicle. Myocardium was assayed for LC3-II, with higher expression in the presence of BFA, indicating greater autophagic flux. Summary data, values normalized to Tsc2WT/WT vehicle, data are mean ± s.d., n = 4 biologically independent experiments; two-way ANOVA: P < 1 × 10⁻⁶ for BFA effect, P = 0.003 for Tsc2 genotype effect, and P = 0.002 for interaction; Sidak’s pairwise multiple comparisons test: *P = 0.0008 versus Tsc2WT/WT vehicle, ¹P < 1 × 10⁻⁶ versus Tsc2S1365E/WT vehicle and P = 0.008 versus Tsc2S1365A/S1365A + BFA, ²P = 0.05 versus Tsc2WT/WT + BFA.
## Extended Data Table 1 | Cardiac morphometry and function analyses

### Mice Aged 2-3 Months

|                      | TSC2 WT | n  | TSC2 S1365E | p-value | TSC2 WT | n  | TSC2 S1365A | p-value |
|----------------------|---------|----|-------------|---------|---------|----|-------------|---------|
| Body Weight (g)      | 25.0 ± 4.1 | 11 | 23.9 ± 2.9  | 0.60    | 22.5 ± 3.3 | 12 | 21.8 ± 2.4  | 0.60    |
| Heart Weight (mg)    | 119.7 ± 23.6 | 6  | 113.6 ± 12.4 | 0.88    | 117.0 ± 24.7 | 5  | 114.9 ± 12.7 | 0.88    |
| Left Ventricular Weight (mg) | 84.7 ± 17.6 | 6  | 80.5 ± 9.6   | 0.44    | 81.2 ± 13.4 | 5  | 75.4 ± 4.8   | 0.44    |
| Lung Weight (mg)     | 141.7 ± 14.5 | 6  | 128.3 ± 13.0 | 0.72    | 133.4 ± 15.0 | 5  | 130.2 ± 10.8 | 0.72    |
| Tibial Length (mm)   | 17.8 ± 0.8  | 6  | 17.6 ± 0.5   | 0.30    | 18.2 ± 0.8   | 5  | 17.8 ± 0.3   | 0.30    |
| HW/LT                | 6.7 ± 1.1       | 6  | 6.4 ± 0.6    | 0.92    | 6.4 ± 1.1    | 5  | 6.5 ± 0.6    | 0.92    |
| LVW/TL               | 4.7 ± 0.8       | 6  | 4.6 ± 0.5    | 0.57    | 4.5 ± 0.6    | 5  | 4.3 ± 0.3    | 0.57    |
| LuW/TL               | 7.9 ± 0.6       | 6  | 7.3 ± 0.7    | 1.00    | 7.3 ± 0.7    | 5  | 7.3 ± 0.5    | 1.00    |
| Heart Rate (bpm)     | 680.4 ± 56.6    | 11 | 690.0 ± 67.9 | 0.60    | 675.8 ± 21.4 | 12 | 683.2 ± 42.9 | 0.60    |
| %Ejection Fraction   | 82.0 ± 1.5      | 11 | 83.4 ± 3.7   | 0.74    | 82.1 ± 1.6   | 12 | 81.8 ± 2.6   | 0.74    |
| %Fractional Shortening | 57.6 ± 3.0   | 11 | 59.5 ± 4.4   | 0.76    | 57.7 ± 1.9   | 12 | 57.4 ± 2.9   | 0.76    |

### Mice Aged 9-12 Months

|                      | TSC2 WT | n  | TSC2 S1365E | p-value | TSC2 WT | n  | TSC2 S1365A | p-value |
|----------------------|---------|----|-------------|---------|---------|----|-------------|---------|
| Body Weight (g)      | 30.2 ± 3.0 | 6  | 28.5 ± 3.5  | 0.37    | 29.6 ± 3.1  | 8  | 29.2 ± 4.2  | 0.37    |
| Heart Weight (mg)    | 163.4 ± 25.6 | 6  | 147.9 ± 26.3 | 0.31    | 159.5 ± 25.0 | 8  | 167.3 ± 32.2 | 0.31    |
| Left Ventricular Weight (mg) | 119.2 ± 23.1 | 6  | 109.3 ± 27.1 | 0.50    | 125.7 ± 25.6 | 8  | 134.8 ± 33.9 | 0.50    |
| Lung Weight (mg)     | 149.2 ± 20.9 | 6  | 130.8 ± 17.4 | 0.11    | 141.4 ± 24.5 | 8  | 161.5 ± 37.5 | 0.11    |
| Tibial Length (mm)   | 19.7 ± 1.4   | 6  | 19.6 ± 1.3  | 0.90    | 19.1 ± 1.1  | 8  | 19.1 ± 1.2  | 0.98    |
| HW/LT                | 8.3 ± 0.8      | 6  | 7.5 ± 0.9    | 0.14    | 8.3 ± 0.9   | 8  | 8.7 ± 1.4    | 0.51    |
| LVW/TL               | 6.0 ± 0.9      | 6  | 5.5 ± 1.0   | 0.37    | 6.5 ± 1.0  | 8  | 7.0 ± 1.6   | 0.50    |
| LuW/TL               | 7.6 ± 0.7      | 6  | 6.7 ± 0.7   | 0.04    | 7.4 ± 0.9  | 8  | 8.4 ± 1.7   | 0.15    |
| Heart Rate (bpm)     | 704.2 ± 37.5   | 6  | 693.6 ± 25.3 | 0.56    | 671.3 ± 74.3 | 8  | 659.3 ± 86.4 | 0.56    |
| %Ejection Fraction   | 77.2 ± 2.9     | 6  | 77.0 ± 2.7  | 0.90    | 80.8 ± 1.9  | 8  | 68.2 ± 24.6 | 0.90    |
| %Fractional Shortening | 52.3 ± 3.1   | 6  | 52.1 ± 2.8  | 0.89    | 52.3 ± 2.2  | 8  | 46.5 ± 19.4 | 0.89    |

Cardiac morphometry and function were analysed in Tsc2S1365E and Tsc2S1365A knock-in mice and their respective age-matched littermate controls. Top, results for 2–3-month-old mice. Bottom, results for 9–12-month-old mice. The results show negligible effects of the Tsc2 knock-in mutations in the basal state at both ages. b.p.m., beats per min. P-values are from two-sided unpaired Student’s t-tests.
### Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

#### Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

| n/a | Confirmed |
|-----|-----------|
|     | ![ ]      |
| ![ ] | The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement |
| ![ ] | An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| ![ ] | The statistical test(s) used AND whether they are one- or two-sided |
| ![ ] | Only common tests should be described solely by name; describe more complex techniques in the Methods section. |
| ![ ] | A description of all covariates tested |
| ![ ] | A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons |
| ![ ] | A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) |
| ![ ] | For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted |
| ![ ] | Give P values as exact values whenever suitable. |
| ![ ] | For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings |
| ![ ] | For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes |
| ![ ] | Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated |
| ![ ] | Clearly defined error bars |
| ![ ] | State explicitly what error bars represent (e.g. SD, SE, CI) |

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### Software and code

Policy information about availability of computer code

**Data collection**

All data were collected on individual hardware devices (e.g. Visualsonics Vevo 2100 for echocardiographic images, or Licor Odyssey for Western blot, Biorad CFX384 qPCR machine for mRNA expression. No stand-alone open source or commercial software was used for data collection.

**Data analysis**

Mouse genetic background assessment utilized SNaP-MaP™ and Map-Synth™ performed at the Geisel School of Medicine at Dartmouth University, Hanover, NH; Western blot images were performed using Licor Image Studio Software 3.1, fluorescent images using tandem LC3-GFP-RFP probe was analyzed using Image J (Version 1.52a), echocardiographic images were performed using VisualSonics Vevo 2100, qPCR data was processed using Microsoft Excel, and statistical analysis using Graphpad Prism Version 7.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.
Data

Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The authors declare that [the/all other] data supporting the findings of this study are available within the paper [and its supplementary information files]. Requests for original computer files containing the reported data are available from the corresponding author upon reasonable request.

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☐ Life sciences  ☐ Behavioural & social sciences  ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Data exclusions | Replication |
|--------------|----------------|-------------|
| If variance in the measured variable was known, then sample size for the assay was based either on known variance in the measured variable and thus power calculation of the requisite sample size to determine at least a 20% different in the mean variable (echo studies for example, or immunoblot), at 80% power and 95% confidence. For parameters where a variance was not previously known, studies were replicated at least 6 times, and based on these results, variance assessed and replicates performed to achieve a similar level of power and confidence. | Data were not excluded | The following list identifies what was done to assure reproducibility for each experiment in this study: Figure 1A, 1B: Based on multiple mice (n=24 total, assigned randomly to 4 groups), all data used and results highly reproducible. Figure 1C: Individual cell images for each condition with overall variance depicted and all data displayed. Results were highly reproducible. Figure 1E (upper) Highly reproducible result, performed by several laboratory members. Figure 1E(lower), 1F, 1G: Based on all mice used in study displayed in Figure 1A. No data excluded. Results highly reproducible. Figure 1H: Sample size of n=12 per group provided sufficient power. Random selected tissues, no bias. Tissue all processed identically. Figure 1I: Reproduced x3 with no disparities, also reproduced with another mutation (x3, n=6 total). Very consistent. Figure 1J: All technical replicates and biological replicates have demonstrated the identical findings. This was repeated x6. Figure 2A: Very strong group differences, and study now shown in entirety using two different serine mutations, fully reproducible. Figure 2B: All cell culture studies used, and data shown in paper demonstrates identical responses with either of the serine mutations. Figure 2C: Same situation as in Figure 1C, all cell data shown, despite variance between cells, group differences are marked, and reproduced. Figure 2D: Data consistent with other results previously shown (without siRNA to Rheb), and technically certified runs used. Very reproduced. Figure 2E: Data from 5 separate experiments, 2 independent investigators with very consistent results. Very reproducible. Figure 2F: All data used from population studied in time course and drug trial. Figure 2G,2H: Experiment confirmed in 3D despite interanimal variance, statistical differences are marked. Figure 2I: Nearly binary results of assay that was very reproducible, all gels for this study were utilized. Figure 2J: Performed in all surviving animals at end-of-study, no bias, and consistent with sample size in other assays from mouse study. Figure 2K: While this entire study was done once, the results were marked, and sample size sufficient to power detection. Figure 2L: Very reproducible results as demonstrated by highly disparate effects among groups. Figure 2M: Example echocardiography which is consistent with mean results demonstrated in Figure 4D. Figure 2N: Same animals used to generate imaging and functional data were used for immunoblots. Results marked and reproducible. Figure 2O: Same explanation as provided for Figure 4F. Extended Data 1A-1C: Same experiment shown in Figure 1A, 1B, and same explanation applies. Extended Data 1D: Experiment performed once, though results were striking and differences highly significant. This is further consistent with subsequent data shown in Figure 3F in which protein aggregation was assayed in a different manner. Extended Data 2A: Experiment performed once, all data shown. Little variance in independent samples, supports reproducibility. Extended Data 2B: Experiment performed with multiple biological replicates, PCR results show marked differences. Extended Data 2C: Relates to Figure 1E Upper. Extended Data 2D: Performed multiple times by multiple investigators, panel here shows high concordance of results, reproducible. Extended Data 3A: Given variance, we performed study with larger sample size, providing robust power for interpretation. Extended Data 3B: While there is some inter-group variance, overall analysis shows very consistent result in each group. Extended Data 3C: Relates to Figure 1E lower. |
Randomization

Mouse models involving drug treatment were randomized up front prior to the procedure being performed - pressure overload with or without concomitant drug treatment. Individuals providing drug (oral or IP), or performing echocardiographic analysis were blinded to group allocation. All controls were performed in littermates using heterozygote crossing strategies to yield 25% WT, 50% heterozygote, and 25% homozygote. Male and female mice were equally included in this study and were age-matched and weight-matched.

Blinding

We blinded all in vivo functional analysis involving genotype and/or drug treatment - with the echo-sonographer assessing images blinded to both. Molecular analysis (western blots, immunoprecipitation, etc) was performed by several individuals, where the experiment and sampling was obtained by one, isolates analyzed by another. In these instances, the individuals were aware of the experimental groups involved.

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☑ | ☑ Unique biological materials |
| ☑ | Antibodies |
| ☑ | Eukaryotic cell lines |
| ☑ | Palaeontology |
| ☑ | Animals and other organisms |
| ☑ | Human research participants |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☑ | ChIP-seq |
| ☑ | Flow cytometry |
| ☑ | MRI-based neuroimaging |

Unique biological materials

Policy information about availability of materials

Obtaining unique materials

The study involves two novel custom developed KI mouse models. We will provide these mice to other investigators under an MTA, once an initial study is published. These models will be subject to an anticipated patent that was already filed and is related to this discovery.

Antibodies

Antibodies used

The following primary antibodies were used in this study: phospho-Akt (S473) #9271 lot 14 used at 1:1,000, Akt #9272 lot 28 used at 1:1,000, phospho-70 S6K (T389) #9205 lot 21 used at 1:1,000, p70 S6K #9202 lot 20 used at 1:1,000, phospho-4EBP1 (S65) #9451 lot 14 used at 1:1,000, 4EBP1 #9452 lot 12 used at 1:1,000, phospho-Ulk-1 (S757) #1420 clone D706U lot 4 used at 1:1,000, Ulk-1 #8054 clone DBH5 lot 1 used at 1:1,000, phospho-FOXO1/3 #9464 lot 7 used at 1:1,000, FOXO1 #2880 clone C29H4 lot 11 used at 1:500, phospho-NRDG-1 #3217 clone D98G11 lot 3 used at 1:1,000, NRDG-1 #9395 clone D6C2 lot 1 used at 1:1,000, GAPDH #2118 clone 14C10lot 10 used at 1:1,000, Rheb #13879 clone E1G1R lot 1 used at 1:1,000, phospho-TSC2 (S1387) #5584 lot 5 used at 1:1,000, TSC2 #3612 clone D93F12lot 5 used at 1:1,000, and α-tubulin #8051 clone D93F12lot 5 used at 1:1,000, and α-tubulin #8051 clone D93F12lot 5 used at 1:1,000.

All antibodies used were either well established in the literature, for which validation in the model system (mouse or human)
Validation

All of the commercial antibodies were previously well described and validated. The antibody for S1365 TSC2 phosphorylation obtained from NovoPro had not been previously reported. Validation was performed in MEFs lacking the protein, with re-expression of the protein with and without site mutations at the S1365 that impact phosphorylation of the residue (e.g. S1365A which prevents it), and then with and without kinase activation (PK). These validation studies are presented in the manuscript. Each commercial antibody was validated by the manufacturer as noted at their website. The information for this validation is listed for each antibody used in the study in a separate Antibody Validation Table Document.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

We used mouse embryonic fibroblasts (standard cell line, or MEFs with TSC2 knocked-out) provided from the laboratory of Brendan Manning. These have been used in many prior studies and are well established models. We also generated a new HEK TSC2 KO cell line and present these methods and line details in the revision. Other cells are primary isolated neonatal ventricular myocytes from rat - so not a cell line per se. Methods for this preparation are well established and referenced.

Authentication

We did not perform specific authentication procedures for the cells used in this study, as the cell lines are well established and widely used, and the primary isolations are not cell lines.

Mycoplasma contamination

Our cultured cells are assessed for evidence of bacterial contamination in a more general manner, as part of maintenance of our incubator systems and being sure we have no infectious contamination. However, we did not specifically test for mycoplasma.

Commonly misidentified lines

None were used.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals

For all in vivo studies the mice were aged around 2-3 months at the time of pressure overload or sham surgeries. Wild type C57 black mice were purchased from Charles River. TSC2 SA and SE knock in mice were designed by our lab and generated at the Johns Hopkins Mouse Transgenics Core using CRISPR/Cas9 technology. They are also raised in the C57 black background. All protocols and procedures were approved by the Johns Hopkins IUCAC. The studies were in compliance with all ethical regulations. Both male and female mice were utilized in this study.

Wild animals

The study did not involve the use of wild animals.

Field-collected samples

The study did not involve the use of samples collected from the field.

Human research participants

Policy information about studies involving human research participants

Population characteristics

De-identified human tissue was made available to study through an agreement between Johns Hopkins (David Kass, PI) and University of Pennsylvania (Ken Margulies, PI). Patient information including demographics, clinical diagnosis and hemodynamic, treatment, and other relevant clinical parameters are provided. Left ventricular tissue from non-failing control hearts are obtained from organ donors whose heart was not used for transplantation (Gift of Life). The research is approved by both Johns Hopkins and Univ. Pennsylvania respective IRBs. There were two cohorts of samples. For the non-failing controls, the age was 52.8 ± 15.4, 6 males, 6 females, LV function in normal range, and no clinical history of heart failure; for the heart failure group, the mean age was 51.3 ±12.1, 8 males, 4 females, all with severe dilated non-ischemic cardiomyopathy who then all underwent subsequent cardiac transplantation.

Recruitment

The samples are obtained from either deceased patients or patients receiving a heart transplant - the latter are alive and recruited from those subjects receiving transplantation at the University of Pennsylvania. The particular samples reported in this study were randomly taken from a database of many such patients in each group, selected purely based on their inclusion in either the control or heart failure cohort. Informed consent was obtained from failing heart human tissue donors. The family or legal representative provided consent for organ harvesting from deceased donor controls.