Pathological hypertrophy amelioration by PRAS40-mediated inhibition of mTORC1

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Mechanistic target of rapamycin complex 1 (mTORC1), necessary for cellular growth, is regulated by intracellular signaling mediating inhibition of mTORC1 activation. Among mTORC1 regulatory binding partners, the role of Proline Rich AKT Substrate of 40 kDa (PRAS40) in controlling mTORC1 activity and cellular growth in response to pathological and physiological stress in the heart has never been addressed. This report shows PRAS40 is regulated by AKT in cardiomyocytes and that AKT-driven phosphorylation relieves the inhibitory function of PRAS40. PRAS40 overexpression in vitro blocks mTORC1 in cardiomyocytes and decreases pathological growth. Cardiomyocyte-specific overexpression in vivo blunts pathological remodeling after pressure overload and preserves cardiac function. Inhibition of mTORC1 by PRAS40 preferentially promotes protective mTORC2 signaling in chronic diseased myocardium. In contrast, strong PRAS40 phosphorylation by AKT allows for physiological hypertrophy both in vitro and in vivo, whereas cardiomyocyte-specific overexpression of a PRAS40 mutant lacking capacity for AKT-phosphorylation inhibits physiological growth in vivo, demonstrating that AKT-mediated PRAS40 phosphorylation is necessary for induction of physiological hypertrophy. Therefore, PRAS40 phosphorylation acts as a molecular switch allowing mTORC1 activation during physiological growth, opening up unique possibilities for therapeutic regulation of the mTORC1 complex to mitigate pathologic myocardial hypertrophy by PRAS40.

The mechanistic target of rapamycin (mTOR) kinase is a central cellular hub that couples nutrient sensing and growth factor signaling to cell growth and survival. mTOR signaling is often deregulated in cardiac diseases, and altered growth kinetics, metabolic changes, and increased susceptibility to cell death are characteristics of dysfunctional cardiomyocytes accumulating after cardiac damage. Pharmacological inhibition of mTORC1 with rapamycin improves cardiac function after pressure overload, myocardial infarction, and in genetic hypertrophic cardiomyopathies (1–3). Rapamycin improves cardiac function in patients after kidney transplantation and inhibits the development of cardiac hypertrophy (4), however no established therapeutic regime targets mTOR specifically in cardiomyocytes. As a consequence of the ubiquitous role of mTOR in cell biology, off-target and systemic effects limit clinical use of rapamycin in patients.

mTOR exists in two distinct complexes, mTORC1 and mTORC2 (5, 6). Regulatory-associated protein of mammalian target of rapamycin (Raptor) and Proline Rich AKT Substrate of 40 kDa (PRAS40) are specific to mTORC1. Although regulatory mechanisms activating mTORC1 are relatively well understood, those regulating mTORC2 are less characterized. mTORC2 is defined by assembly with rapamycin-insensitive companion of mTOR (Rictor) that has a regulatory role in the insulin signaling cascade and AKT activation (7). PRAS40 interacts with Raptor and inhibits mTORC1 kinase activity (8, 9). PRAS40 contains two proline-enriched stretches at the amino terminus and an AKT consensus phosphorylation site (RXRXXS/T) located at Thr246. Phosphorylated PRAS40 dissociates from mTORC1 in response to growth factors, insulin, glucose, and nutrients, thereby releasing the inhibitory function on mTORC1 (10, 11). Mutation of Thr246 to alanine inhibits AKT-mediated phosphorylation that is important to relieve the inhibitory action of PRAS40 on mTORC1 (9, 12). mTORC1 is an upstream regulator of PRAS40–Ser^{183} phosphorylation, which is important for binding of PRAS40 to Raptor (10, 11). PRAS40 regulates cellular growth and survival in vitro (13), but PRAS40 involvement in the regulation of growth in any tissue remains unexplored. As typical for other well-characterized regulators of cardiac growth and survival [e.g., AKT, proto-oncogene serine/threonine-protein kinase Pim-1 (PIM1), calcineurin], PRAS40 was initially discovered in noncardiac cells, but the relevance of PRAS40 in the myocardium has been overlooked. PRAS40 is widely expressed in various human and mouse tissues, with particularly high expression in the heart (14). In this study we demonstrate the cardioprotective effects of PRAS40-mediated inhibition of mTORC1 using a clinical relevant cardiac gene therapy.

Results

PRAS40 Is Expressed in Myocytes and Is Phosphorylated After Pressure Overload. PRAS40 is highly expressed in cardiomyocytes, with insulin treatment and subsequent AKT induction prompting PRAS40 phosphorylation at residues Thr^{246} and Ser^{183} together with activation of mTORC1 and mTORC2 (Fig. 1*A*).

PRAS40 is phosphorylated in response to insulin (Fig. 1*B*) and localized in the nucleus as shown by immunofluorescence, consistent with previous reports (15, 16). PRAS40 interaction with mTORC1 was assessed using proximity ligation assays (PLAs) in vitro. PRAS40 association with RAPTOR decreased after treatment with insulin (neonatal cardiomyocytes, NRCMs) in isolated neonatal myocytes and diminished binding of PRAS40 to mTOR after stimulation with insulin was confirmed by immunoprecipitation (Fig. S1*A*). The function of PRAS40 as an mTORC1 inhibitor can be negated by phosphorylation (thereby releasing mTORC1 inhibition) under conditions supporting increased mTORC1 activity, such as pressure overload induced by transaortic constriction (TAC). PRAS40 expression and phosphorylation were determined at multiple time points after TAC by immunoblot analysis. $PRAS^{T246}$ and $PRAS40^{S183}$ phosphorylation increased as early as 30 min after TAC (Fig. 1*C*), correlating with activation of mTORC1 as measured by phosphorylation of S6Kinase (S6K) as well as Ribosomal S6 protein (RibS6) and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP) 4EBP, mTORC2 (measured by phosphorylation of AKT), and activation of fetal gene expression (Fig. S1*B*). PRAS40 phosphorylation diminished over time and decreased in the chronic phase of TAC response (5–8 wk postsurgery; Fig. 1*C*).

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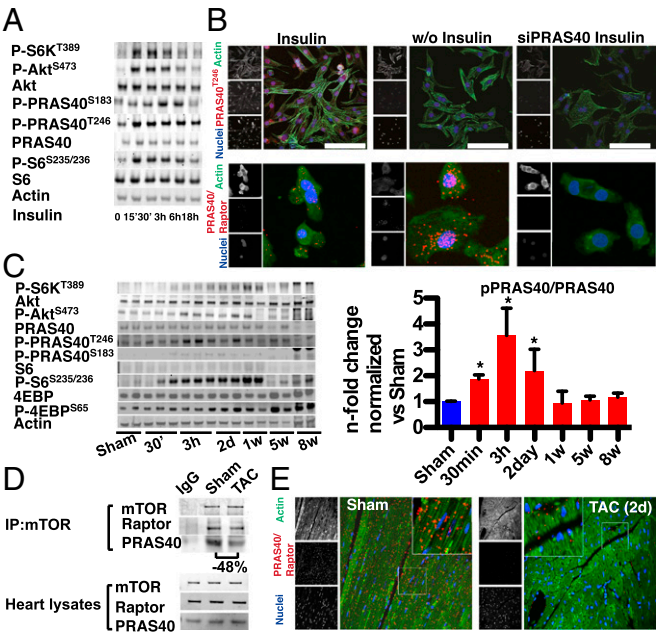

Fig. 1. PRAS40 is expressed in myocytes and is phosphorylated after pressure overload. (*A*) Time course after insulin stimulation. PRAS40 is phosphorylated after stimulation with insulin (100 nM). (*B*, *Upper*) Immunofluorescence of myocytes stained for pPRAS (red), actin (green), and nuclei (blue). PRAS40 is phosphorylated after insulin stimulation (20 min). Knockdown of PRAS40 with siRNA confirms specificity. (Scale bar, 150 μm.) (*B*, *Lower*) PLA (in red) in isolated myocytes under starvation conditions and after stimulation with insulin. Each red dot represents one interaction of PRAS40 with Raptor. Fewer interactions are present after insulin stimulation, indicating PRAS40 release from mTORC1. (*C*) Immunoblot showing a time course after TAC in 10-wk-old mice surgery for PRAS40 expression and phosphorylation. $*P < 0.05$ versus sham. (*D*) mTOR immunoprecipitation after sham or TAC surgery. (*E*) PLA in paraffin-embedded sections after TAC surgery. Fewer interactions are present after TAC, indicating PRAS40 release from mTORC1.

These data suggest that AKT-driven inactivation by phosphorylation of PRAS40 contributes early after TAC to activation of mTORC1. PRAS40 binding to mTOR decreased by 48% after TAC as assessed by immunoprecipitation (Fig. 1*D*). Decreased binding of PRAS40 to mTOR after pressure overload was confirmed by PLA in paraffin sections in vivo (Fig. 1*E*).

PRAS40 protein and phosphorylation levels were assessed in human failing myocardium using explanted cardiac tissue. PRAS40 protein expression was 2.1-fold higher together with increased PRAS40^{T246} phosphorylation in failing human myocardium relative to normal control myocardium, consistent with increased mTORC1 activation in failing hearts (Fig. S1*C*). Increased brain natriuretic peptide (BNP) levels confirmed failing myocardium (Fig. S1*C*).

PRAS40 Blocks Pathological Growth in Vitro. Because PRAS40 is an inhibitor of mTORC1, which is the major cellular regulator of growth, overexpression should block pathological hypertrophy. Therefore, the role of PRAS40 in hypertrophy was determined using adenoviral vectors carrying FLAG-tagged cDNAs encoding wild-type PRAS40 and a phospho-dead myc-tagged mutant (threonine to alanine, PRAS40TA). Consequences of PRAS40 overexpression for cardiomyocyte hypertrophy were examined using NRCMs infected with adenoviruses encoding PRAS40 or control followed by stimulation with phenylephrine (PE) for 24 h. PRAS40 phosphorylation did not significantly increase, indicating that PRAS40 phosphorylation is not required for the stimulation of mTORC1 signaling after PE, in line with previous reports (Fig. S2*A*) (17). PRAS40 overexpression inhibited PE-induced hypertrophy (Fig. 2 *A* and *B*) as assessed by cell surface area (CSA) measurements, comparable to the effect of rapamycin. As expected, the TA mutant blocked hypertrophic growth to a similar extent as the wild-type PRAS40 (Fig. 2*B*). mTOR kinase activity was inhibited in a dose-dependent manner by recombinant PRAS40 protein in vitro (Fig. 2*C*). Mechanistically, PRAS40 blocks increased mTORC1 (assessed by decreased phosphorylation of mTORC1 downstream targets S6K, RibS6, and 4EBP) activation in NRCM in response to PE (Fig. 2 *D* and *E*). The ratio of phosphorylated PRAS40–total PRAS40 was lower compared with control cells, which results in decreased mTORC1 activity in vitro (Fig. S2*A*). Expression of Raptor or phosphorylation of mTOR kinase remained unchanged after PRAS40 overexpression. Hypertrophic gene markers after PE showed decreased mRNA levels for atrial natriuretic peptide (ANP) by 91.3% and BNP by 52.4%, resulting from PRAS40 overexpression (Fig. S2*B*). To test if silencing of PRAS40 expression is sufficient to induce growth in NRCM, PRAS40-specific siRNAs were transfected into NRCMs. PRAS40 mRNA transcription was lowered to 9% of control levels within 48 h after silencing (Fig. S2*C*). Protein synthesis increased after PRAS40 silencing, which was associated with increased sensitivity to lower concentrations of PE and increased cellular size, whereas the maximal cell size was not affected after PRAS40 silencing (Fig. S2 *D* and *E*). Thus, PRAS40 overexpression blocks pathological hypertrophic growth in vitro by blocking mTORC1, whereas reduction of PRAS40 expression sensitizes cardiomyocytes to PE.

PRAS40 Blocks Pathological Growth in Vivo. An adeno-associated virus stereotype 9 (AAV9) virus expressing PRAS40 under regulatory control of myosin light chain (MLC) promoter in vivo was generated for cardiomyocyte-specific expression and to determine the effects of PRAS40 overexpression on pathological growth in vivo (Fig. S3*A*). PRAS40-overexpressing mice were then subjected to TAC. Staining for PRAS40 using the FLAG-tag confirmed overexpression in myocytes (Fig. S3*B*). Heart size was increased significantly in control mice at 1 and 5 wk after TAC (Fig. 3 *A* and *B*). Notably, hypertrophic growth was inhibited in hearts of PRAS40-overexpressing mice, as evidenced by a cross-sectional area of myocytes (Fig. 3*C*). PRAS40 blocked mTORC1 activation in response to TAC at 1 wk (Fig. 3*D*) as well as 5 wk after TAC (Fig. S3 *C* and *D*). Furthermore, molecular markers of hypertrophy such as ANP and BNP were induced after TAC in control mice after 1 wk (4.1-fold and 2.5-fold) and 5 wk (7.3-fold and 14.3-fold), but this increase was blocked in mice overexpressing PRAS40 (Fig. 3*E*). Consistent with blocked remodeling, PRAS40 TAC-challenged hearts exhibit decreased perivascular fibrosis and decreased collagen1 transcription (Fig. 3*F* and Fig. S3*D*) relative to their control TAC-challenged counterparts. Serial echocardiography was performed to measure cardiac function after TAC. Anterior wall thickening confirmed control hearts underwent remodeling starting 1 wk after challenge (Fig. 4*A* and Tables S1 and S2). In comparison, PRAS40 animals did not show significant increases in wall thickness up to 4 wk after surgery. In addition, left ventricular enlargement was completely prevented and systolic function preserved in PRAS40 animals (Fig. 4*B*).

PRAS40 Prevents Deterioration of Cardiac Function. Although the above results are a classic example of a prevention study, preemptive intervention does not reflect the clinical situation where patients most likely will be identified when they already have developed cardiac hypertrophy. To test the hypothesis that PRAS40 is protective when injected after banding when initiation of hypertrophy was confirmed by echocardiography, TAC mice were injected with PRAS40 or control virus 1 wk after banding (Fig. 5*A*). Hearts of control mice started to dilate and failed 4 wk after banding assessed with serial echocardiography. Cardiac function deteriorated for up to 8 wk after TAC in control animals,

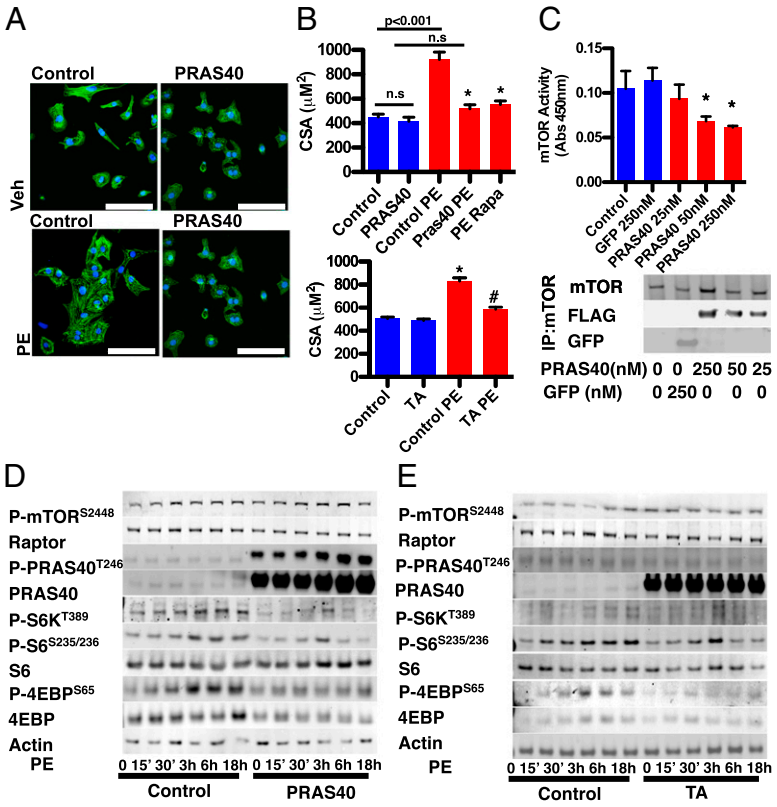

Fig. 2. PRAS40 blocks pathological growth in vitro. (*A*) Representative images of control and PRAS40-treated cardiac myocytes stained with an α-actinin antibody. Sytox is used as a stain for the nuclei. (Scale bar 150 μm.) (*B*) Individual CSA measurements from control, PRAS40, PRAS40TA, rapamycin-treated NRCMs, and those treated with PE (100 μM for 24 h) ($n = 4$ independent experiments, $^{*}P < 0.05$ vs. control PE, $^{\#}P < 0.05$ vs. control PE). (C) ELISA-based activity assay for measuring the kinase activity of mTOR. Recombinant protein blocks dose-dependent mTOR. Immunoprecipitates containing the specified concentrations of PRAS40 or GFP were performed and analyzed by immunoblotting for the indicated proteins. (*D*) Immunoblot showing that PRAS40 blocks activation of mTORC1–S6K1 pathway in NRCMs in response to PE. (*E*) Immunoblot showing that PRAS40TA blocks activation of mTORC1–S6K1 pathway in NRCMs in response to PE.

whereas PRAS40-treated animals showed stabilization of cardiac function and chamber diameter starting at 4 wk after banding. Superior heart function in PRAS40 animals was confirmed by hemodynamic measurement 8 wk after surgery (Fig. 5*B*). Chronic mTORC1 activation results in decreased mTORC2 signaling (5) that might be rescued by PRAS40 overexpression. Indeed, PRAS40-treated mice showed increased phosphorylated Akt (pAKT) levels and increased insulin Receptor Substrate–1 (IRS-1) expression 8 wk after TAC (Fig. 5*C*). Control animals showed signs of congestive heart failure with increased lung weight/body weight ratio (LW/BW), which was prevented in PRAS40-treated mice (Fig. 5*D*). PRAS40 overexpression led to reduced molecular markers of hypertrophy (Fig. 5*E*) and fibrosis (Fig. S3*E*). Importantly, exposure to pressure overload was similar, as the pressure across the aorta was not statistically different among the groups of mice subjected to TAC (Fig. S3*F*). Taken together these data support the idea that PRAS40 can block pathological remodeling and protect cardiac function after TAC, even when injected after initiation of pathological hypertrophy.

PRAS40 Does Not Prevent Physiological Growth. Physiological cardiac hypertrophy occurring after chronic exercise training is distinct

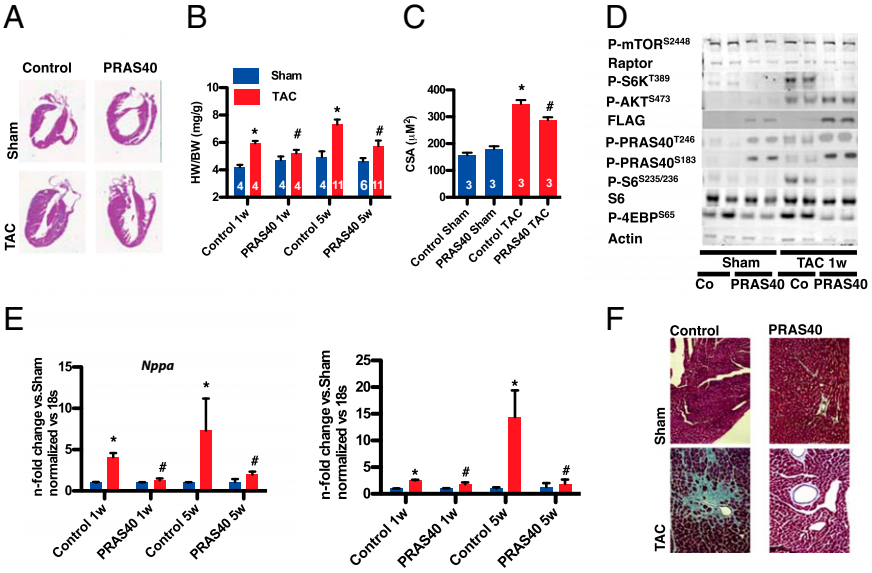

Fig. 3. PRAS40 blocks pathological growth in vivo. (*A*) Gross morphology with H&E staining of either control virus or PRAS40-injected mouse hearts after either sham or TAC surgery in 10-wk-old mice. (*B*) Heart weight (HW) to BW ratio (HW/BW) in control and PRAS40 mice either 1 or 5 wk after sham or TAC surgery. The number of mice per group is indicated within the bar ($^{*}P < 0.01$ versus control sham; $^{\#}P < 0.05$ versus control TAC). (*C*) CSA in control and PRAS40 mice 5 wk after sham or TAC surgery ($^{*}P < 0.01$ versus control sham; $^{\#}P < 0.05$ versus control TAC). (*D*) Immunoblots of whole heart lysates 1 wk after sham or TAC surgery. (*E*) ANP and BNP transcription 1 and 5 wk after sham or TAC surgery ($^{*}P < 0.01$ versus control sham; $^{\#}P < 0.05$ versus control TAC). (*F*) Masson–Trichrome staining from control and PRAS40-treated hearts. Error bars indicate means ± SEM.

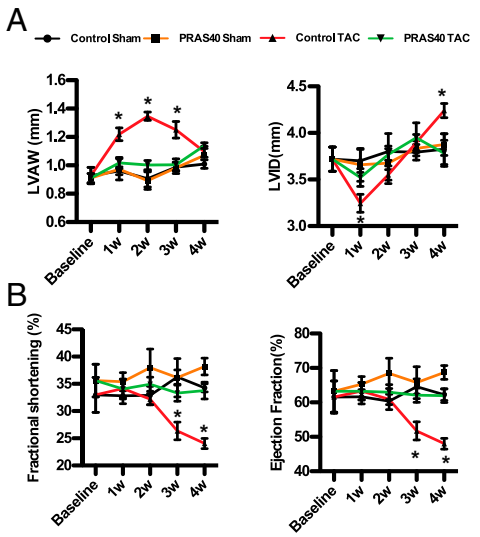

Fig. 4. PRAS40 protects against cardiac dysfunction. (*A*) PRAS40-treated mice (10-wk-old) are resistant to pressure-overload–induced hypertrophy. Line graphs representing weekly echocardiographic assessment of control or PRAS40 sham and TAC-banded hearts for anterior wall dimension (left ventricular wall, LVAW), end-diastolic dimension (left ventricular diameter, LVID), (*B*) percentage of fractional shortening (FS), and ejection fraction (EF) (*$P < 0.05$ vs. control TAC; $n = 4$ per sham and 11 per TAC group). Error bars indicate means ± SEM.

from pathological hypertrophy insofar as being reversible and characterized by normal heart function. Physiological hypertrophy is associated with activation of the phosphatidylinositide 3-kinase (PI3K)–AKT pathway (18, 19), which should strongly promote PRAS40 phosphorylation. Activation of the PI3K–AKT pathway with insulin in NRCM resulted in a small but significant increase in CSA (1.24-fold). Myocytes overexpressing wild-type PRAS40 also responded to insulin with a 1.3-fold increase in cell size (Fig. 6*A*). The ratio of phosphorylated PRAS40–total PRAS40 increased in both control cells and PRAS40-overexpressing NRCMs (Fig. S4*A*). Conversely, rapamycin and PRAS40TA blocked cell size increases, indicating PRAS40 phosphorylation is necessary for physiological growth in vitro. Mechanistically, PRAS40 overexpression inhibited mTOR kinase activity, whereas inhibition of mTOR with PRAS40TA impaired insulin sensitivity (Fig. 6*A*). Less exogenous PRAS40 (detected with the FLAG-tag) was bound to mTOR after treatment with insulin, indicating that sufficient PRAS40 is released from mTORC1 to allow mild physiological growth (Fig. S4*B*). Phosphorylation of RibS6 and 4EBP was not completely blocked by PRAS40 overexpression, whereas the TA mutant completely blocked the phosphorylation (Fig. 6*B*), indicating that strong AKT activation phosphorylates sufficient overexpressed PRAS40 to allow mTORC1 activation. These in vitro findings were confirmed by overexpression of PRAS40 or phospho-dead mutant PRAS40TA with AAV9 in vivo. Physiological hypertrophy can be studied in vivo by a voluntary running wheel experiment. Mild hypertrophy was induced without altering cardiac function as assessed by echocardiography or without increase of hypertrophic gene markers such as ANP (Fig. 6*C* and Fig. S4 *C* and *D*). In agreement with our in vitro data, physiological hypertrophy was not blocked by PRAS40 overexpression, but was inhibited by PRAS40TA (Fig. 6*C*). Exercised mice showed increased AKT phosphorylation compared with sedentary animals (Fig. 6*D*). PRAS40^{T246} phosphorylation that was nearly undetectable in sedentary control mice was markedly increased by exercise in both control mice and PRAS40-overexpressing mice, whereas PRAS40^{S183} phosphorylation remained unchanged. Whereas RibS6 was unphosphorylated in PRAS40 sedentary mice, exercise induced phosphorylation of RibS6 in PRAS40 mice, indicating activation of mTORC1. Raptor expression and mTOR phosphorylation were unaltered after PRAS40 overexpression (Fig. 6*D*), consistent with the in vitro data (Fig. 2*D*). Citrate synthase activity, an index of muscle oxidative capacity and hence physical training, was measured in mixed gastrocnemius muscle of mice that underwent running training to assure that all animals were running to a similar extent (Fig. S4*E*). This supports the idea that phosphorylation of PRAS40 is necessary during physiological hypertrophic growth, as overexpression of a phospho-dead mutant blocks physiological growth, whereas overexpression of wild-type PRAS40 does not prevent physiological hypertrophy, because AKT phosphorylates sufficient PRAS40 to allow activation of mTORC1.

Discussion

mTORC1 is activated in various cardiac diseases including myocardial infarction, hypertrophic growth, genetic cardiomyopathies, and diabetic cardiomyopathies, and rapamycin treatment improves cardiac function in experimental murine cardiomyopathic models. However, chronic administration of rapamycin exhibits systemic side effects, whereas PRAS40 can be delivered to target mTORC1 specifically in myocytes. Selective inhibition of mTORC1 in cardiomyocytes protects myocardium from pathological remodeling via manipulation of PRAS40. Therefore, PRAS40 represents a unique molecular tool to inhibit pathological remodeling.

PRAS40 initially identified as a 14–3–3 binding protein (14) was subsequently found to serve as an mTORC1 inhibitor (8, 9). Nutrients and growth stimuli result in phosphorylation of PRAS40, leading to dissociation of PRAS40 from the complex and relieving

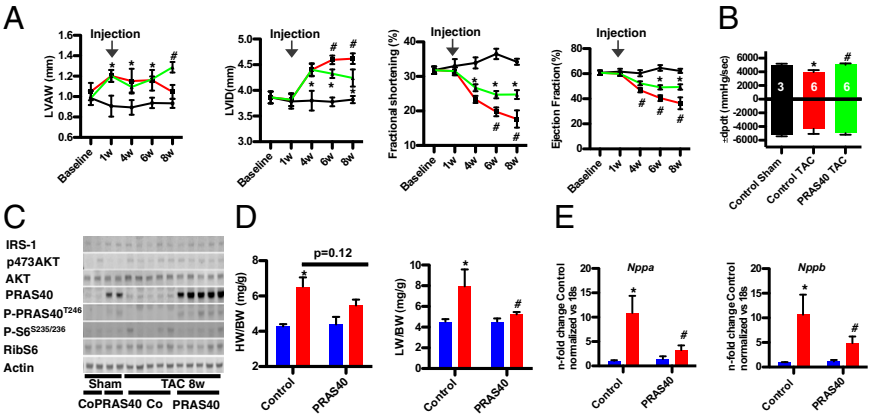

Fig. 5. PRAS40 prevents deterioration of cardiac failure after pressure overload. (*A*) Before injection of PRAS40 or control AAV, echocardiography was performed on each group of mice (10-wk-old). Line graphs representing serial echocardiographic assessment of sham and TAC-banded hearts for LVAW, LVID, FS, and EF (*$P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. control TAC). (*B*) In vivo hemodynamic assessment of control and PRAS40 hearts 8 wk after sham or TAC operation (*$P < 0.05$ versus control sham; $^{\#}P < 0.05$ versus control TAC). (*C*) Immunoblots of whole hearts 8 wk after TAC. (*D*) HW/BW and LW/BW in mice 8 wk after surgery (*$P < 0.01$ versus control sham; $^{\#}P < 0.05$ versus control TAC). (*E*) Nppa and Nppb transcription 8 wk after surgery (*$P < 0.01$ versus control sham; $^{\#}P < 0.05$ versus control TAC). Error bars indicate means ± SEM.

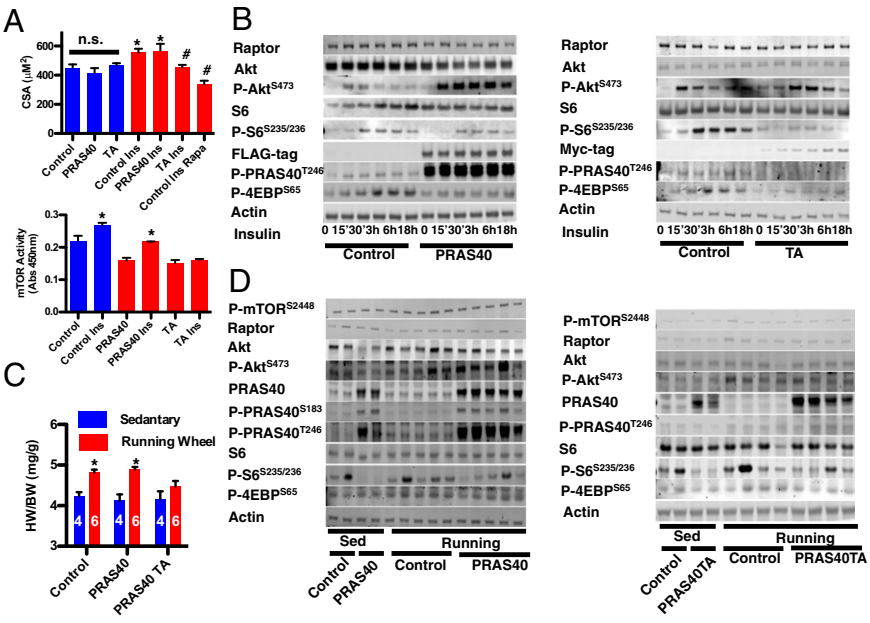

Fig. 6. PRAS40 overexpression allows physiological growth. (*A*, *Upper*) CSA measurements from control, PRAS40, PRAS40TA-infected, or rapamycin-treated (20 nM) NRCMs treated and untreated with insulin (100 nM for 24 h) ($n = 4$ independent experiments, $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ versus control Ins). (*A*, *Lower*) ELISA-based activity assay for measuring the kinase activity of mTOR. PRAS40 overexpression blocks mTOR activity. Ins, insulin. (*B*) Cell lysates of control, PRAS40, and PRAS40TA overexpressing myocytes were analyzed by immunoblotting for the levels of the indicated proteins and phosphorylation states. (*C*) HW/BW in control, PRAS40, and PRAS40TA mice (14-wk-old) after 4 wk of exercise. (*D*) Immunoblot of whole heart lysates of the indicated groups 4 wk after sedentary and exercise. Exercise increase of AKT and PRAS40^{T246} phosphorylation. Error bars indicate means ± SEM.

inhibitory constraint. Consistent with findings in noncardiac cells (9, 10), PRAS40 blocks cellular growth in myocytes by inhibition of mTORC1 (Fig. 2). Interestingly, PRAS40 expression also increases in human failing hearts, suggestive of compensation for ongoing hypertrophic growth stimuli. However, knockdown of PRAS40 does not increase cellular growth at baseline conditions, but sensitizes myocytes to growth stimuli with PE. In addition, we cannot rule out the possibility that PRAS40 may influence cell growth by an alternate mechanism independent of mTOR. We have recently initiated creation of PRAS40 null mice to further understand the roles of PRAS40 in cardiac development and growth, which will be the subject of further studies.

Cardiac remodeling during pathological hypertrophic growth is not adaptive in the long term, and clinical studies support beneficial effects of inhibiting chronic remodeling (20–22). Therefore, maladaptive responses have often been targeted with a remarkable number of possible therapeutic targets for treatment of cardiac diseases. However, development of new drugs for heart failure with depressed heart function (with the possible exception of the bradycardic agent ivrabradine) (23) and for patients with heart failure with preserved function (22) have not readily translated from bench to bedside. Most existing therapies target outside–in signaling in cardiac cells, but are limited in effectiveness. Consequently, targeting intracellular signaling might have better therapeutic potential than existing therapies. Recent advancements in the development of AAV vectors resulted in the first clinical trial with AAV-based cardiac gene transfers (24, 25) and manipulation of sarcoplamic reticulum calcium ATPase (SERCA2a) activity improves cardiac function after TAC (26). AAV-mediated gene therapy represents a unique approach to treat and prevent pathological hypertrophy (27, 28). Gene therapy with cardiac-specific expression of PRAS40 completely prevented pathological hypertrophy and ventricular remodeling after TAC, preventing deterioration of cardiac function when injected after surgery, a time point where systolic function is still preserved, but hypertrophy is initiated. Because protein expression following AAV-based gene delivery takes 2–3 wk, initial chamber dilatation and hypertrophy was not repressed, whereas treatment with rapamycin in a former study blocked hypertrophy after TAC (1).

The PI3K–AKT signaling pathway is a critical mediator of physiological hypertrophy (19). The results show that AKT activation during exercise is sufficient to release enough PRAS40 from mTORC1 to allow mild physiological growth. Corroborating evidence showed that overexpression of a phospho-dead mutant PRAS40 blocked physiological hypertrophy, indicating that phosphorylation of PRAS40 is necessary for physiological growth.

Collectively, the results indicate that PRAS40 is a potent and critical mediator of cardioprotection. Effects of PRAS40 were tested in two relevant models, demonstrating that selective mTORC1 inhibition with PRAS40 is a unique therapeutic option to prevent chronic pathological remodeling. Given that hyperactivation of mTORC1 also occurs in diabetic and aging hearts, future studies will focus on whether PRAS40 is also beneficial in other cardiac diseases. Studying PRAS40 biology in the myocardium will potentially reveal unique ways to treat molecular signaling in high-impact research areas including response to pathologic injury, repair, survival, and age-related cardiac dysfunction.

Materials and Methods

Mice, Surgery, and Cardiac Function Analysis. All experiments were performed in 2-d- to 52-wk-old male C57BL/6 mice unless otherwise indicated. Seven-week-old male C57BL/6 mice were purchased from Jackson Labs. Tail vein injection of either control AAV, PRAS40 AAV, or PRAS40TA were performed at 7 wk of age. At 10 wk of age, male mice underwent TAC (TAC model) or a sham operation as previously described (29, 30). A voluntary running regimen was conducted for 4 wk, as previously described (31). For echocardiography, mice were anesthetized with 2% (vol/vol) isoflurane and scanned using a Vevo770 imaging system (Visual Sonics), as previously described (32). Closed chest hemodynamic assessment was performed on anesthetized mice (33). For in vivo injection of insulin, we injected mice i.p. with PBS or insulin (1 U/kg per BW for 1 h) after overnight starvation. Approval from the San Diego State University Institutional Animal Care and Use Committee was obtained for all animal studies.

Isolation and Primary Culture of Neonatal and Adult Ventricular Cardiomyocytes. Isolation and primary culture of neonatal and adult ventricular cardiomyocytes were prepared by standard procedures. Cells were treated with 100 μM PE or insulin (100 nM) for indicated time points. For analysis of hypertrophy, cells were treated with PE or insulin for 24 h.

Adenoviral Constructs and siRNA. To generate recombinant adenoviruses, the human and mutated human PRAS40 cDNAs were subcloned into the pShuttle–CMV vector using the AdEasy XL Adenoviral Vector system (Stratagene) as previously described, with additional details provided in *SI Text* (29).

AAV9 Generation and Systemic in Vivo AAV9 Cardiac-Targeted Gene Transfer Protocol. In vivo cardiac-targeted PRAS40 expression in normal mouse hearts was obtained by using tail vein injection of an AAV9 harboring the PRAS40

gene or PRAS40TA mutant driven by a cardiomyocyte-specific CMV–MLC2v0.8 promoter as previously described (34).

Sample Preparation, Immunoblotting, RT-PCR, and Immunoprecipitation. Whole-heart, isolated myocytes and human heart lysates were prepared as described previously, with additional details provided in *SI Text* (29). Immunoblots from isolated cells or tissue were conducted as previously described (30). A complete list of antibodies and primers is provided in Tables S3 and S4. RNA was isolated using the Quick RNA MiniPrep kit (ZymoResearch) according to the manufacturer's protocol. We generated cDNA and carried out real-time PCR using the cDNA preparation kit and SYBR real-time PCR (Biorad) according to the manufacturer's protocol. We calculated differences using the $\Delta\Delta C(T)$ method. Immunoprecipitation of mTOR and PRAS40 was carried out as described below using a previously published protocol (8). PRAS40 or GFP cDNAs were transfected into HeLa cells and the proteins purified using immobilized FLAG-antibody resin. Proteins were eluted from the resin with 50 mg/mL FLAG peptide and stored on ice until use. In the experiments using recombinant protein, PRAS40 or GFP was incubated for 30 min to the precipitated mTORC before measuring the mTOR kinase activity.

mTOR Kinase Activity Assay. mTOR kinase activity was measured with the K-LISA mTOR Activity Kit (EMD–Millipore), whicht uses a p70S6K–GST fusion protein as a specific mTOR substrate using the manufacturer's protocol.

Histology and Staining. Immunocytochemistriy was performed using standard procedures, with additional details provided in *SI Text*. Sections were cut and deparaffinized using standard procedures. Immunostaining on paraffin-embedded hearts was performed as described previously in detail (35). Sections were also used to visualize cardiomyocyte cell membrane by staining with tetramethyl rhodamine isothiocyanate–conjugated wheat-germ agglutinin (Sigma-Aldrich).

PLA. PLAs to detect protein–protein interactions in situ were performed as previously described (36).

Measurement of Citrate Synthase Activity. Citrate synthase activity in skeletal muscle was measured using the Citrat Synthase ELISA kit (Abcam) using the manufacturer's protocol.

Statistical Analysis. Statistical analysis was performed using Student *t* test, and ANOVA as appropriate, with Tukey or Bonferroni post hoc tests. All data were analyzed with GraphPad Prism 5.0 (Graphpad Software Inc.; P values < 0.05 were considered significant).

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