Differential effects of various genetic mouse models of the mechanistic target of rapamycin complex I inhibition on heart failure

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Abstract Inhibition of mammalian target of rapamycin complex I (mTORC1) by rapamycin improves cardiac function in both aging and heart failure. While the protective mechanisms are not fully understood in mammals, they are presumably mediated through metabolic regulation and suppression of protein translation by reduced phosphorylation of 4EBP1, a target of mTORC1. Using transverse aortic constriction (TAC)

and $G\alpha_q$ overexpression-induced heart failure models, we examined the effect of cardiac-specific heterozygous deletion (het) of Raptor, a component of mTORC1, and cardiac-specific transgenic overexpression of wild type or phosphorylation site mutant 4EBP1. In wild-type mice with TAC-induced heart failure, quantitative shotgun proteomics revealed decreased abundance of proteins of mitochondrial metabolism and increased

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abundance of proteins in oxidative stress response, ubiquitin, and other pathways. The Raptor heterozygous deletion ameliorated both TAC- and $G\alpha_q$ overexpression-induced heart failure and the associated proteomic remodeling, especially those pathways involved in mitochondrial function, citric acid cycle, and ubiquitination. In contrast, transgenic overexpression of either wild type or mutant 4EBP1 aggravated TAC and $G\alpha_q$, consistent with reduced adaptive hypertrophy by suppression of protein translation, in parallel with adverse remodeling of left ventricular proteomes. Partial mTORC1 inhibition by Raptor heterozygous deletion ameliorates heart failure and is associated with better preservation of the mitochondrial proteome; however, this effect does not appear to be mediated through suppression of protein translation by increased 4EBP1. Increased activity of 4EBP1 reduced adaptive hypertrophy and aggravated heart failure, suggesting that protein translation is essential for adaptive hypertrophy in pressure overload.

Keywords Aging · Heart failure · Nutrient signaling · mTOR · Proteomics

Introduction

Mammalian target of rapamycin (mTOR) regulates metabolism and cellular growth in response to nutrient status and growth factor signaling. Inhibition of the mTOR pathways by calorie restriction or rapamycin have been shown to mediate the beneficial effects of lifespan extension in several aging model organisms (Harrison et al. 2009; Kenyon 2010; Miller et al. 2011). In the heart, both calorie restriction and rapamycin attenuate cardiac hypertrophy and heart failure in aging (Dai et al. 2014) and in pressure overload models of heart failure (McMullen et al. 2004a; Niemann et al. 2010; Shinmura et al. 2011; Shioi et al. 2003; Taffet et al. 1997). The mechanisms by which TOR exerts its effects on aging involve the modulation of protein synthesis (Steffen et al. 2008) and ribosomal biogenesis through mTOR complex 1 (TORC1) and its downstream targets, the translational repressor 4EBP1 and ribosomal p70S6 kinase (p70S6K) (Guertin and Sabatini 2007; Sengupta et al. 2010). Active TORC1 phosphorylates p70S6K, which accelerates ribosome biogenesis, and phosphorylates 4E binding protein 1 (4EBP1), which de-represses cap-dependent initiation

of translation. Thus, inhibition of TORC1 suppresses ribosome biogenesis through suppression of p70S6K and suppresses protein translation through increased activity of 4EBP1. In *Drosophila*, activation of 4EBP has been shown to mediate lifespan extension and the protective effect of dietary restriction on cardiac aging (Wessells et al. 2009; Zid et al. 2009).

Rapamycin protects against pathological hypertrophy and heart failure in both transverse aortic constriction (TAC) and a genetic model of cardiomyopathy (SFig. 1) (McMullen et al. 2004a; Shioi et al. 2003). However, the downstream signaling mediating the benefits of rapamycin or mTOR inhibition is not well understood. The beneficial effect of rapamycin in mouse models of hypertrophy and heart failure is accompanied by suppression of activated phosphorylated ribosomal S6 protein and eIF4E, which are phosphorylated in pressure overload (Gao et al. 2006). However, to the best of our knowledge, all of the reported genetic mouse models with suppressed mTOR complex I function have detrimental effects. Mice with ubiquitous or cardiac-specific homozygous deletion of mTOR are lethal, indicating that mTOR is required for growth and proliferation of embryonic as well as cardiac development (Gangloff et al. 2004; Murakami et al. 2004). Cardiac-specific ablation of Raptor (a component of mTORC1) impairs adaptive hypertrophy and leads to heart failure (Shende et al. 2011). Ablation of mTOR in the adult mouse myocardium results in a fatal dilated cardiomyopathy, mediated through accumulation of 4EBP1 (Zhang et al. 2010).

Downstream of mTORC1 (Fig. 1b), deletion of both p70S6K1 and p70S6K2 in mice had no impact on the development of cardiac hypertrophy after transverse aortic constriction (TAC), exercise-induced hypertrophy, or cardiac hypertrophy in IGF1 receptor or PI3K mutants (McMullen et al. 2004b). These results indicate that P70S6K signaling alone is not critical for the induction of cardiac hypertrophy, despite the fact that mice with deletion of P70S6K1 have increased life span and resistance to age-related pathologies (Selman et al. 2009). Another important downstream pathway of mTORC1, 4EBP, has been shown to mediate the beneficial effect of caloric restriction and mTOR inhibition in *Drosophila* cardiac aging (Wessells et al. 2009).

In the current study, we investigated the potential downstream mechanisms underlying the beneficial effects of mTORC1 inhibition using two mouse

models of heart failure. Based on the data from *Drosophila*, we tested the hypothesis that cardiac-specific overexpression of WT 4EBP1 or a constitutively active 4EBP1 mutant protein might be beneficial in murine heart failure models and compared this with the effect of modest inhibition of TORC1 by heterozygous deletion of Raptor. We found that Raptor heterozygous deletion ameliorates heart failure in response to either TAC or $G\alpha_q$ overexpression, consistent with the beneficial effect of rapamycin. Surprisingly, overexpression of either 4EBP1 WT or 4EBP1 mutant protein aggravated heart failure phenotypes, suggesting that suppression of protein translation does not mediate the benefit of mTORC1 inhibition in the murine heart.

Methods

Mice with genetic suppression of mTOR complex I pathway (Fig. 1b)

Raptor $\pm$ heterozygous deletion (Raptor het) were generated using a Raptor gene trap knockout ES cell line obtained from Bay Genomics (BG143) on a B6

background. The 4EBP1 transgenic mice were generated as described. Briefly, 4EBP1 was inserted into a CAGGS expression cassette preceded by a floxed STOP cassette (Tsai et al. 2015). Two versions of the 4EBP1 transgene were made: the wild-type 4EBP1 (4EBP1-Tg) and a phosphorylation site 4EBP1-A37/A46 mutant version (4EBP1-mut) (Li et al. 2002). These mice were bred to cardiac-specific MHC-cre mice on B6 strain. All animal experiments were approved by the University of Washington Institutional Animal Care and Use Committee.

Transverse aortic constriction surgery and echocardiography

Transgenic male mice at ~ 12 weeks of age were injected with tamoxifen to excise the floxed STOP sequence to create cardiac-specific transgenic mice (Fig. 1a). At ~ 16 –17 weeks, 6–12 mice of cardiac-specific Raptor het, 4EBP1-Tg, and 4EBP1-mut and WT littermates underwent TAC surgery 3 weeks after tamoxifen induction of the genetic modification. TAC was performed as described (Kim et al. 2008; Tarnavski et al. 2004), under ketamine (130 mg/kg, IP) and xylazine (8.8 mg/kg, IP) anesthesia, supported by a

Fig. 1 **a** Experimental design. **b** The mTOR complex I pathways. **c** Quantitative PCR of Raptor mRNA expression * $p < 0.05$ vs. WT. **d** Western blot analysis of total and phosphorylated 4EBP1.

Bar charts show mean $\pm$ SEM; P/T = ratio of phospho- to total protein. * $p < 0.05$ vs. WT

ventilator. Briefly, the skin was incised at the 3th~6th intercostal space, followed by successive layers of subcutaneous tissue/intercostal muscle dissection. A sterile ligature was passed around the exposed aortic arch; then, a blunted 26 gauge needle was placed on top of the aorta; the ligation was tied around the needle, and then, the needle was immediately removed. Echocardiography was performed at baseline and at the end of experiments (4 weeks after TAC) using a Siemens Acuson CV-70 equipped with 13-MHz probe, as described (Dai et al. 2009). Briefly, isoflurane 0.5% mixed with O₂ was used to provide adequate sedation but minimal cardiac suppression during echocardiography. M-mode, conventional and Tissue Doppler echocardiography, and functional calculations were performed according to American Society of Echocardiography guidelines. Myocardial performance index (MPI) is the ratio of isovolemic time (isovolemic contraction and relaxation time) to effective aortic ejection time. Higher MPI indicates worse myocardial performance, as longer time is needed during the isovolemic (ineffective) phase to cope with the change in pressure. Mice were harvested 4 weeks after TAC by cervical dislocation. The heart was removed immediately, rinsed quickly in cold saline, and then flash-frozen in liquid nitrogen.

Sample preparation and analysis by mass spectrometry

Six mice from each experimental group were processed for proteomics. Frozen left ventricular apex tissues were homogenized in cold buffer (250 mM sucrose, 1 mM EGTA, 10 mM HEPES, 10 mM Tris-HCl pH 7.4). The lysates were centrifuged at 800×g for 10 min to get rid of the debris, then suspended in 0.1% RapiGest (Waters Corporation, Milford, MA) and boiled. The samples were sequentially treated with 5 mM DTT, then 15 mM iodoacetamide, and trypsinized at 37 °C for 2 h, followed by 200 mM HCl, and spun down at 20,000g. The supernatant was washed using a Waters Oasis MCX sample extraction column. One to two micrograms of digested protein sample was loaded onto the ultra-performance liquid chromatography and mass spectrometry (UPLC-MS/MS), using a Waters nano Acquity LC system and a Thermo Scientific LTQ-FT Ultra. MS/MS spectra were searched by SEQUEST (ver.27) against a mouse IPI database (v3.57). Peptide spectrum match false discovery rates were determined by the Percolator algorithm (Kall et al. 2007) with a *q*-value threshold of 0.01. In order to reduce the effect of

chromatographic drift that may occur during sample analyses, we applied Topograph software for chromatographic alignment (Dai et al. 2013).

Proteomics data analysis

In order to determine statistically significant changes of proteins between experimental groups, we used a linear model of peptide abundance to calculate fold changes of proteins between experimental groups in the same manner as a two-sample *t* test using the R/Bioconductor software (Gentleman et al. 2004). Bioconductor package *q*-value was used to calculate statistically significant genes while controlling the estimated false discovery rate. The canonical pathways were generated through the use of Ingenuity Pathway Analysis (IPA, Ingenuity Systems, www.ingenuity.com), as previously described (Dai et al. 2013). The raw data from MS/MS and extended supplementary files (including a table showing log₂ fold-differences in protein abundance for sham, Raptor het-TAC, 4EBP1-Tg-TAC, and 4EBP1-mut-TAC compared to WT-TAC) are available at <https://chorusproject.org/pages/blog.html#/957>. In order to view the data, a free account must be obtained by following the instructions on the Chorus Project website.

Western blot analysis

Antibodies used for the Western blots were phospho-4EBP1, 4EBP1, phospho-Akt, Akt, phospho-AMPK, AMPK, phospho-ULK and ULK (all from Cell Signaling), phospho-FOXO (Fisher), FOXO (Abcam), Donkey anti-rabbit, and anti-mouse secondary antibodies (Thermo Scientific).

Polysome analysis

Ventricular tissue was homogenized in a Zomzely buffer (250 mM sucrose, 50 mM Tris-HCl, 100 mM KCl, 12 mM MgCl₂, and 50 mg/ml cyclohexamide); then, 1% deoxycholate was added, followed by centrifugation at 13,200x rpm for 10 min. The supernatant was collected, and optical density was measured at 260 nM. Twenty to twenty-five optical density units in 1 ml homogenization buffer were added to a 10.8-ml linear 15–60% sucrose gradient containing 2× Zomzely gradient buffer (with addition of 200 µg/µl cyclohexamide). Samples were sedimented in an SW41 Ti rotor

(Beckman) at 39,000 rpm at 4 °C for 2 h. Gradient fractions were collected from the top. Absorption was measured at 254 nM. This data was recorded using WinDaq software. Polysome profiles were quantified by measuring the area under the peaks corresponding to 40S, 60S, 80S, and polysome peaks, and dividing them by total area.

Results

The experimental design and the genetic mouse models with modified mTORC1 signaling are summarized in Fig. 1a, b. Using this design, we aimed to recapitulate the beneficial effects of rapamycin in both TAC and the Gαq overexpression-induced heart failure model (SFig. 1) using three genetic models of reduced mTOR signaling. These transgenic mouse models were cardiac-specific heterozygous deletion of Raptor (Raptor het), cardiac-specific transgenic overexpression of wild-type 4EBP1 (4EBP1-Tg), and cardiac-specific transgenic expression of mutant 4EBP1 (4EBP1-A37/A46, abbreviated as 4EBP1-mut), in which the mutant phosphorylation sites are not subject to TOR kinase inhibition (i.e., a constitutively active form of 4EBP1) (Li et al. 2002; Tsai et al. 2015).

Quantitative PCR shows that the expression of Raptor in heart tissue of Raptor het mice was 0.56 ± 0.05 of the WT level, consistent with the hemizygous expression of the cardiac Raptor gene (Fig. 1c). Western blots of total 4EBP1 protein demonstrate a 9.3-fold overexpression in 4EBP1-Tg and a 3.3-fold overexpression in 4EBP1-mut hearts, while the Raptor het has only a ~28% increase in total 4EBP1 (Fig. 1d). Relative to that seen in WT mice, the ratio of phospho- to total 4EBP1 is 0.3 in 4EBP1-Tg and 0.26 in 4EBP1-mut, while in Raptor het it is 0.89 (Fig. 1d).

Pressure overload-induced heart failure is ameliorated by heterozygous deletion of Raptor but aggravated by overexpression of 4EBP1 WT or mutant protein

At baseline, cardiac structure or function of Raptor heterozygous deletion, wild-type 4EBP1, or mutant 4EBP1 is normal as measured by echocardiography. No significant effect on systolic or diastolic function was observed in 11-month-old 4EBP1-Tg or 4EBP1-mut mice, ~8 months after tamoxifen induction (Stab1).

After 4 weeks of TAC-induced pressure overload, echocardiography demonstrated significantly decreased fractional shortening (FS) in WT mice from 53.2 ± 2.2 to $32.3 \pm 1.4\%$ ($p < 0.001$, Fig. 2a). Heterozygous deletion of cardiac Raptor significantly ameliorated TAC-induced systolic dysfunction, with FS of $37.7 \pm 1.1\%$ ($p = 0.005$ compared with WT-TAC). In contrast, cardiac-specific 4EBP1-Tg mice exhibited significantly worse FS (25.6 ± 2.4 , $p = 0.04$) when compared with WT-TAC. Cardiac-specific 4EBP1-mut mice had the worst systolic function among all groups (FS, $19.8 \pm 2.9\%$, $p = 0.006$ vs. WT-TAC). As shown in Fig. 2b, TAC significantly impaired myocardial performance (increased MPI) in WT mice, which was slightly attenuated by Raptor het. Cardiac-specific 4EBP1-mut mice showed significantly aggravated MPI, when compared with WT-TAC. There was a ~27% increase in LV end-diastolic dimension by M-mode echocardiography after TAC in WT mice ($p < 0.01$, Fig. 2c), indicating LV dilatation. While Raptor het and 4EBP1-Tg mice showed no significant difference in left ventricular end-diastolic dimension (LVEDD) from WT mice, 4EBP1-mut dramatically increased LVEDD by 60% ($p < 0.01$, Fig. 2c), indicating a dramatic dilatation of LV. Figure 2d shows significantly increased LV interventricular septum (IVS) and left ventricular posterior wall (LVPW) thickness after TAC in WT mice. While Raptor het and 4EBP1-Tg showed slight attenuation of TAC-induced increase in wall thickness, 4EBP1-mut-TAC mice had wall thickness comparable with that of the sham-treated animals, which was significantly thinner than WT-TAC ($p < 0.05$), indicating the absence of compensatory hypertrophy after TAC-induced pressure overload. Figure 3a shows representative image of hearts after TAC. While Raptor het-TAC mice have smaller hearts than WT-TAC, both 4EBP1-Tg-TAC and 4EBP1-mut-TAC mice have larger/dilated hearts. As shown in Fig. 3b, TAC significantly increased normalized crude heart weights in WT mice by 71% ($p < 0.01$). When the normalized heart weight of each group was compared with that of WT-TAC, Raptor het-TAC was ~13% lower, 4EBP1-Tg-TAC was similar, and 4EBP1-mut-TAC was ~14% higher. Figure 4c demonstrates that normalized lung weight significantly increased by 84% in WT mice after TAC ($p < 0.01$), indicating lung congestion secondary to congestive heart failure. When the normalized lung weight of each group was compared with WT-TAC, Raptor het-TAC was ~

Fig. 2 Echocardiographic measurements in TAC-induced heart failure. **a** Systolic function measured by percent fractional shortening (FS%) showed a dramatic decline in WT-TAC at 4 weeks, which was significantly ameliorated in Raptor het-TAC, but significantly aggravated in both 4EBP1-Tg-TAC and 4EBP1-mut-TAC. **b** Measurement of myocardial performance index (MPI) showed a significant impairment (increase) in WT-TAC, which was significantly aggravated in 4EBP1-mut-TAC. **c** Left

ventricular end-diastolic dimension (LVEDD) was significantly increased after TAC in WT and further aggravated in 4EBP1-mut-TAC. **d** Both interventricular septum (IVS) and left ventricular posterior wall (LVPW) thickness were significantly increased after TAC in WT. 4EBP1-mut-TAC had significantly thinner IVS and LVPW when compared with WT-TAC. * $p < 0.05$ for sham vs. WT-TAC, # $p < 0.05$ vs. WT-TAC

22% lower ($p < 0.05$), 4EBP1-Tg-TAC was similar, and 4EBP1-mut-TAC was ~28.3% higher.

Pathway analysis of proteome remodeling in pressure overload-induced heart failure and the effect of genetic manipulation of mTOR complex I

Figure 4 displays the heat map of protein abundance of treatment groups, ordered by the significance of the IPA canonical pathway and shown as relative differences from WT-TAC. The majority of proteins involved in mitochondrial dysfunction, glycogen degradation, TCA cycle, and the mitochondrial carnitine shuttle pathway are significantly more abundant in sham as compared with WT-TAC, indicating a significant decrease in these proteins in pressure overload-induced heart failure, consistent with prior reports (Dai et al. 2013; Fang et al. 2013). Raptor het significantly attenuated many of the changes in TAC-induced heart failure, maintaining protein abundances in these pathways at levels comparable with sham. This was confirmed by analysis of the

partial regression of the sham/WT-TAC vs. Raptor het/WT-TAC ratios (eliminating the statistical effect of WT-TAC being common to the comparison), which showed a spearman's correlation of 0.67 between sham and Raptor het protein abundance ($p < 10^{-46}$) (also see SFig. 2). In contrast, the majority of proteins involved in many signaling pathways, including actin cytoskeleton signaling, protein ubiquitin pathway, integrin, and axonal guidance signaling have lower expression in sham, as compared with WT-TAC, indicating a significant increase in these proteins in pressure overload-induced heart failure. Likewise, Raptor het significantly attenuated many of these changes and partially maintained protein abundances in these pathways at levels comparable with sham. In 4EBP1-Tg or 4EBP1-mut hearts, many of the proteins in the top IPA pathways were either not different in these mice after TAC when compared with WT-TAC (nearly white on the heat map) or were changed in different directions from those seen in sham mice. These findings suggest that the 4EBP1 transgenes potentiate some of the adverse proteome

Fig. 3 Pathology of TAC-induced heart failure. **a** Representative images of hearts after TAC in WT, 4EBP1-Tg, 4EBP1-mut transgenic, and Raptor het. Both normalized heart weight (**b**) and lung

weight (**c**) significantly increased in WT-TAC, which were attenuated in Raptor het-TAC but aggravated in 4EBP1-mut-TAC. * $p < 0.05$ vs. sham, # $p < 0.05$ vs. WT-TAC

remodeling of TAC-induced heart failure (Fig. 4, bottom two rows), consistent with their aggravated heart failure phenotype. The pattern of protein changes in 4EBP1-mut transgenic TAC hearts showed greater differences from sham than did the 4EBP1-Tg-TAC hearts. This is consistent with the greater aggravation of heart failure phenotypes in 4EBP1-mut-TAC than 4EBP1-Tg-TAC.

Confirmation using Gαq overexpressing mice

To confirm the roles of heterozygous deletion of Raptor and 4EBP1-Tg models on heart failure, we crossed these transgenic mice with cardiac-specific Gαq-overexpressing mice to generate double transgenic mice, followed by tamoxifen induction of the het knockout or 4EBP1-Tg expression in 10-week-old mice. Gαq mice have been shown to develop heart failure at approximately 16 weeks of age (D'Angelo et al. 1997; Dai et al. 2011b). As shown in Fig. 5a, the FS was significantly lower in Gαq mice, when compared with WT littermates. Raptor het significantly mitigated the decline of FS in Gαq mice ($p < 0.01$). In contrast, 4EBP1-Tg

significantly aggravated the decline of FS in Gαq mice ($p < 0.05$). The significant impairment (increase) of MPI and dilatation of LV end diastolic diameter in Gαq mice were attenuated by Raptor het ($p < 0.05$ for both, Fig. 5b, c). The increase in LV wall thickness in Gαq mice was not observed in the presence of 4EBP1-Tg (Fig. 5d), indicating the absence of cardiac hypertrophy in these mice.

Signaling mechanisms related to mTOR pathways

In sham-treated mice, 4EBP1-Tg has significantly lower AKT phosphorylation (at serine 473) compared with WT (0.54 ± 0.1 , $p = 0.02$), while Raptor het has slightly higher AKT phosphorylation (1.5 ± 0.3 , $p = 0.2$), and significantly higher FOXO phosphorylation (2.2 ± 0.1 , $p = 0.015$) (Fig. 6). TAC-induced heart failure significantly decreased AKT phosphorylation in WT mice (0.6 ± 0.2 , $p = 0.05$). Compared with WT-TAC, both 4EBP1-Tg and 4EBP1-mut hearts have lower AKT phosphorylation (0.39 ± 0.05 , $p = 0.06$ and 0.29 ± 0.04 , $p < 0.05$, respectively), consistent

Fig. 4 Proteomics analysis of failing hearts with genetic inhibition of mTORC1. Heat maps of abundance differences for sham, Raptor het-TAC, 4EBP1-Tg-TAC, and 4EBP1-mut-TAC showing $\log_2$ fold-differences in protein abundance compared with WT-

TAC, ordered by the rank of significance of differences in Ingenuity Pathway Analysis. Red indicates higher and blue indicates lower abundance (see key showing $\log_2$ fold-difference values)

with inactivation of this survival pathway, while Raptor-het hearts had modestly higher AKT phosphorylation (0.88 ± 0.15 vs. WT-TAC, $p = 0.1$). As phosphorylation of AMPK has been shown to be increased in failing hearts (Kim et al. 2012; Tian et al. 2001), both 4EBP1-Tg-TAC and 4EBP1-mut-TAC hearts have higher AMPK phosphorylation (3.15 ± 0.05 , $p = 0.02$ and 1.79 ± 0.04 , $p = 0.11$, respectively) when compared with WT-TAC (1.31 ± 0.17). In contrast, AMPK phosphorylation was unchanged in Raptor het hearts when compared with WT after TAC. Phosphorylation of FOXO slightly (but non-significantly) increased after TAC in 4EBP1-Tg and 4EBP1-mut but was similar in Raptor-het, when compared with WT-TAC. Another downstream pathway of mTORC1 is Unc-51-Like autophagy-activating kinase 1 (ULK1), which activates the autophagy pathway. While there was no significant change in phosphorylation ratio of ULK1 caused by TAC or in various genetic models, total ULK1 was significantly increased by TAC in both WT and

Raptor-het; however, this increase was not observed in both 4EBP1-Tg and 4EBP1-mut (SFig. 3).

The effect of genetic models on cardiac protein translation

Since suppression of mTORC1 is expected to inhibit cap-dependent translation through disinhibition of 4EBP1 (Fig. 1b), we performed polysome profiling to assess translation state in the hearts. As shown in Fig. 7a, there is a significantly lower ribosome loading of polysomes in the hearts of 4EBP1-mut transgenic mice compared with WT controls, made clearer by examining the trend of ribosome loading of polysomes (Fig. 7b, $p = 0.0074$). Compared with WT littermate controls, the hearts of 4EBP1-Tg mice have significantly higher fractions of 2 or 3 ribosome-loaded polysomes, but lower fractions of > 5 polysomes, suggesting a mild suppression of translation at baseline. Figure 7c shows that the hearts of the Raptor het have indistinguishable

Fig. 5 Echocardiography in $G\alpha q$ overexpressing mouse models with heart failure. **a** Fractional shortening (FS%) significantly decreased in $G\alpha q$ mice at 16 weeks of age, which was significantly ameliorated in $G\alpha q$ /Raptor heterozygotes, but significantly deteriorated in $G\alpha q$ /4EBP1-Tg mice. **b** Myocardial performance index (MPI) showed a significant increase in $G\alpha q$, which was significantly attenuated in the $G\alpha q$ /Raptor heterozygote but was

significantly aggravated in $G\alpha q$ /4EBP1-Tg mice. **c** Left ventricular end-diastolic dimension (LVEDD) was significantly increased in $G\alpha q$ and ameliorated in $G\alpha q$ /Raptor heterozygous mice. **d** Both interventricular septum (IVS) and left ventricular posterior wall (LVPW) thicknesses were increased in $G\alpha q$ mice, though the difference only reached statistical significance for the IVS. * $p < 0.05$ for WT vs. $G\alpha q$, # $p < 0.05$ vs. $G\alpha q$

polysome profiles when compared with WT controls, suggesting the extent of translational suppression in the Raptor het heart is small, if any.

Discussion

Rapamycin has been shown to inhibit angiotensin II-induced protein synthesis in cardiomyocytes (Sadoshima et al. 1995), rejuvenate and reverse cardiac aging (Dai et al. 2014), suppress and regress TAC-induced cardiac hypertrophy (Shioi et al. 2003), and improve systolic function and regress left ventricular fibrosis in mice with hypertrophy and heart failure (Gao et al. 2006; McMullen et al. 2004a). Both calorie restriction and rapamycin are known to inhibit mTOR complex I (mTORC1), a complex of mTOR and Raptor (Fig. 1b). To date, all of the reported genetic mouse models of mTORC1 suppression have shown

detrimental effects that lead to heart failure, including mice with ubiquitous or cardiac-specific homozygous deletion of mTOR (Gangloff et al. 2004; Murakami et al. 2004), inducible ablation of cardiac mTOR in adult hearts (Zhang et al. 2010), or cardiac-specific ablation of Raptor (Shende et al. 2011). This raises a possibility that the beneficial effect of rapamycin on heart failure is not mediated through its inhibition on mTORC1. Rapamycin is known to have “off target” effects including impaired glucose tolerance and insulin resistance due to inhibition of mTOR complex 2 (Lamming et al. 2012). These negative metabolic effects were diminished after prolonged rapamycin treatment; rapamycin treatment for 20 weeks resulted in better metabolic profiles and enhanced insulin sensitivity, consistent with the life extension benefit in chronic rapamycin treatment (Fang et al. 2013).

Two main downstream pathways of mTORC1 include p70S6K and 4EBP1 (Fig. 1b). Active mTORC1

Fig. 6 Analysis of mTOR and AMPK signaling in failing hearts with genetic inhibition of mTORC1. Western blots for AKT, AMP kinase, FOXO, and ULK-1 in sham- and TAC-treated WT,

4EBP1-Tg, 4EBP1-mut transgenic, and Raptor heterozygous deletion (Raptor-het). * $p < 0.05$ vs. WT in the same group

phosphorylates p70S6K, which accelerates ribosome biogenesis. Active mTORC1 also phosphorylates 4EBP1, which results in its release from the inactive 4EBP1/eukaryotic initiation factor 4E (eIF4E) complex, allowing the mRNA cap binding protein eIF4E to associate with eIF4G and eIF4A to form the active eIF4F complex. This complex is required for cap-dependent translation initiation, the major translation initiation pathway in eukaryotes. Inhibition of mTORC1 by rapamycin suppresses activation (phosphorylation) of ribosomal S6 protein via p70S6K and suppresses cap-dependent translation by maintaining 4EBP1 inhibition of eIF4E (Fig. 1b) (Gao et al. 2006). Of these two downstream targets of mTORC1, inhibition of P70S6K signaling alone is not critical in cardiac hypertrophy and failure, as shown by the absence of effect on

the development of cardiac hypertrophy after TAC, exercise-induced hypertrophy or cardiac hypertrophy in IGF1 receptor or PI3K mutants, in mouse models with deletion of both p70S6K1 and p70S6K2 (McMullen et al. 2004b).

To better elucidate the mechanisms underlying the cardioprotective effect of mTORC1 inhibition, we generated three mouse models of inhibition of mTORC1 signaling: cardiac-specific heterozygous deletion of Raptor (Raptor het), overexpression of wild-type 4EBP1 (4EBP1-Tg), or transgenic expression of constitutively active mutant 4EBP1 (4EBP1-mut). We hypothesized that partial reduction of Raptor by heterozygous deletion, which attenuates but does not completely abolish mTORC1 signaling, might recapitulate the beneficial effect of rapamycin

Fig. 7 Polysome profiles. Normalized abundance of polysome fractions of hearts showing extent of ribosome loading (abscissa) in cardiac-specific 4EBP1-mut (a), 4EBP1-Tg (c), and Raptor heterozygous deletion (e). The fractions of loaded ribosomes in

these mice normalized to those in control mice are shown (b, d, f), allowing trend analyses that show a significant decrease in ribosome loading in 4EBP1-mut hearts ($p = 0.0074$). * $p < 0.05$ vs. WT controls

on heart failure. Likewise, we hypothesized that increasing activity of 4EBP1, the other downstream target of mTORC1 that suppresses protein translation, might have similar protective effects, as was previously shown in age-related heart failure in *Drosophila* (Wessells et al. 2009).

In this study, we demonstrated that Raptor heterozygosity ameliorated heart failure induced by both pressure overload (TAC) and overexpression of $G\alpha_q$, and that the beneficial effect was associated with better preservation of the cardiac proteome, especially the proteins involved in mitochondrial function, glucose

metabolism, and the TCA cycle. This favorable proteomic pattern is the same that is seen in the reversal of cardiac aging phenotypes (Dai et al. 2014). To the best of our knowledge, this study is the first to recapitulate the beneficial effect of rapamycin on heart failure using a genetic model with decreased mTORC1 signaling in the heart. Contrary to our initial hypothesis, suppression of protein translation by 4EBP1-Tg or 4EBP1-mut either did not show any benefit or significantly exacerbated heart failure induced by pressure overload (Figs. 2, 3, and 4). The deleterious effect of 4EBP1-Tg was further confirmed in the $G\alpha_q$ overexpressing heart failure mouse model (Fig. 5). In the TAC model, the 4EBP1-mut transgenic mice demonstrated more severe heart failure phenotypes than 4EBP1-Tg, concomitant with stronger suppression of protein translation in the 4EBP1-mut (Fig. 7). This indicates that maintenance of protein synthesis is required for adaptive cardiac hypertrophy and strong suppression of protein translation (as shown by polysome profiles) bypasses the compensatory hypertrophic phase and aggravates heart failure phenotypes. Our observations are also consistent with a prior report using genetic ablation of 4EBP1 to rescue fatal dilated cardiomyopathy in homozygous cardiac mTOR knockout mice (Zhang et al. 2010). Our observed phenotype in 4EBP1-mut hearts was consistent with adverse remodeling of the cardiac proteome, especially a significant decrease in abundance of proteins involved in mitochondrial function. Thus, even though 4EBP1 has been shown to mediate the beneficial effect of caloric restriction and mTOR inhibition in *Drosophila* cardiac aging (Wessells et al. 2009; Zid et al. 2009), this was not observed in murine heart failure models in the current study. Our findings in 4EBP1-Tg or 4EBP1-mut are similar to the previous study of mice with inducible homozygous cardiac ablation of Raptor (Raptor $-/-$) in adult mouse hearts, which showed de-repression of 4EBP1 and aggravated TAC-induced heart failure without adaptive hypertrophy, reduced mitochondrial content, shift in metabolic substrate use, and increased apoptosis and autophagy (Shende et al. 2011). Taken together, these findings indicate that partial inhibition of mTORC1 by either rapamycin (McMullen et al. 2004a; Shioi et al. 2003) (SFig. 1) or the Raptor het is cardioprotective, while complete inhibition of mTORC1 by total disruption of mTOR or Raptor (Gangloff et al. 2004; Murakami et al. 2004; Shende et al. 2011; Zhang et al. 2010) is detrimental, as mTORC1/4EBP1 signaling is required for protein

homeostasis and adaptive cardiac hypertrophy in response to stress.

This study also demonstrated that Raptor het hearts, including those undergoing TAC, have higher AKT phosphorylation, while both 4EBP1-Tg and 4EBP1-mut hearts decreased AKT phosphorylation after TAC, suggesting inactivation of survival pathways in the 4EBP1 models. Consistent with a prior report showing that activated/phosphorylated AMPK is increased during energy crisis in heart failure models (Tian et al. 2001), we showed that AMPK phosphorylation is higher in 4EBP1-Tg and 4EBP1-mut hearts after TAC, compared with WT-TAC.

In summary, heterozygous deletion of Raptor-mitigated TAC and $G\alpha_q$ overexpression induced heart failure, while overexpression of wild-type or mutant 4EBP1 aggravated heart failure by suppressing protein translation, thereby inhibiting compensatory hypertrophy. Global proteomics analyses revealed that the top pathways preserved by partial mTOR inhibition by the Raptor het in TAC-induced heart failure included proteins involved in mitochondrial function and metabolism. As noted above, these are the same pathways that we observed to dominate the protective pattern of rapamycin-induced reversal of age-related proteomic remodeling and heart failure in mice (Dai et al. 2014). There are also the same patterns observed in the beneficial effects of mitochondrial-targeted catalase or mitochondrial-protective peptides on heart failure (Dai et al. 2011a, b, 2012, 2013). Our current study suggests that pharmacologic inhibition of mTOR is likely finely balanced partial suppression of TORC1 activity that is not readily recapitulated by genetic manipulation of downstream targets. This also has significant implications for the development of new rapalogs intended to benefit health span while reducing adverse effects, as these strategies are generally intended to manipulate the balance of downstream effects of mTOR inhibition (Lamming et al. 2013; Schreiber et al. 2019). Alternatively, the proteomic data shown here indicates that pharmacologic targeting of mitochondrial protective pathways may provide novel therapeutics strategies in heart failure, consistent with our previous studies (Dai et al. 2011a, 2013).

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Compliance with ethical standards All animal experiments were approved by the University of Washington Institutional Animal Care and Use Committee.

Conflict of interest The authors declare that they have no conflict of interest.

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