Cardiac mTORC1 Dysregulation Impacts Stress Adaptation and Survival in Huntington’s Disease

Highlights

- Cardiac mHTT expression impairs mTORC1 activity in HD mouse model hearts
- Restoring cardiac mTORC1 activity in HD mice permits cardiac stress adaptation
- Chronic cardiac mTORC1 inhibition is cell-intrinsic, caused by Rheb mislocalization

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In Brief

Child et al. demonstrate that mTORC1 dysregulation is a key molecular mechanism in the Huntington’s disease (HD) heart phenotype. Impaired cardiac mTORC1 activity in HD mouse models requires intrinsic mHTT expression and explains the limited adaptation to cardiac stress.

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Cardiac mTORC1 Dysregulation Impacts Stress Adaptation and Survival in Huntington’s Disease

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SUMMARY

Huntington’s disease (HD) is a dominantly inherited neurological disorder caused by CAG-repeat expansion in exon 1 of Huntingtin (HTT). But in addition to the neurological disease, mutant HTT (mHTT), which is ubiquitously expressed, impairs other organ systems. Indeed, epidemiological and animal model studies suggest higher incidence of and mortality from heart disease in HD. Here, we show that the protein complex mTORC1 is dysregulated in two HD mouse models through a mechanism that requires intrinsic mHTT expression. Moreover, restoring cardiac mTORC1 activity with constitutively active Rheb prevents mortality and relieves the mHTT-induced block to hypertrophic adaptation to cardiac stress. Finally, we show that chronic mTORC1 dysregulation is due in part to mislocalization of endogenous Rheb. These data provide insight into the increased cardiac-related mortality of HD patients, with cardiac mHTT expression inhibiting mTORC1 activity, limiting heart growth, and decreasing the heart’s ability to compensate to chronic stress.

INTRODUCTION

Huntington’s disease (HD) is a fatal, autosomal dominant neurodegenerative caused by CAG-repeat expansion in exon 1 of the huntingtin gene. This mutation creates an expanded polyglutamine tract in the Huntingtin protein (HTT) and results in a mutant form of HTT (mHTT) that disrupts multiple cellular functions (The Huntington’s Disease Collaborative Research Group, 1993). Pathologically, HD is characterized by profound neuronal death in the striatum and other brain regions (Rosas et al., 2001), which causes uncontroled movements (chorea), behavioral abnormalities, and cognitive decline (Walker, 2007). Age of onset inversely correlates with CAG expansion length, and death occurs on average 10–15 years after symptom onset (Wexler et al., 2004). There is currently no cure for HD, but antisense oligonucleotide (ASO) and adeno-associated virus (AAV)-mediated gene therapies to reduce HTT levels in the CNS have demonstrated safety and efficacy in animal models and patients and are progressing in clinical trials (Boudreau et al., 2009; Ionis Pharmaceuticals, 2017; McBride et al., 2011; Stanek et al., 2013). However, HTT expression is ubiquitous (Li et al., 1993; Trottier et al., 1995), and HD affects peripheral tissues with high metabolic demand (Chaturvedi et al., 2010; Mihm et al., 2007; Ribchester et al., 2004). Thus, CNS-specific therapies may unmask peripheral HD phenotypes.

Heart disease is the second leading cause of death in HD patients across multiple populations (Lanska et al., 1988a, 1988b; Sørensen and Fenger, 1992), but the phenotype is still poorly understood. The few clinical studies that examined the heart in HD largely focused on neuronal inputs. Specifically, noninvasive, autonomic function analyses of sympathetic skin responses and heart rate variability measures suggested an abnormal balance between parasympathetic and sympathetic function, with HD patients tending to experience decreased vagal activity and increased sympathetic tone (Bär et al., 2008; Kobal et al., 2004; Sharma et al., 1999). Epidemiological studies report a higher incidence of heart failure in HD patients compared to unaffected age-matched controls (Abildtrup and Shattock, 2013; CDC, 1994), and HD patient heart size is decreased at autopsy (Myers et al., 1998). A recent study specifically looking for cardiovascular disease risk factors found increased risk even in premanifest HD gene carriers (Bellosta Diago et al., 2018). However, the pathogenic mechanisms underlying these phenotypes have remained elusive and may include autonomic circuit degeneration within the CNS, intrinsic cardiac mTORC1 toxicity, and other systemic abnormalities.

In animal models, mHTT expression is associated with adverse cardiac phenotypes. Both Drosophila and mice engineered to express an expanded polyglutamine peptide in heart experience dilated chambers, impaired contractility, and reduced survival (Melkani et al., 2013; Pattison et al., 2008). The transgenic R6/2 and knockin Q150 HD mouse models have dilated ventricles and impaired cardiac function late in disease, coupled with fibrosis, increased apoptosis, altered ganglionic plexus morphology, and conduction abnormalities (Mielcarek et al., 2014b; Mihm et al., 2007; Wood et al., 2012). Cardiac gene expression networks in the R6/2 and Q150 models attribute heart pathology to altered neuronal or other systemic inputs (Mielcarek et al., 2014b). Additionally, the Q175 knockin HD mouse model displays abnormal heart rate variability and...
abnormal baroreceptor response, further implicating the autonomic nervous system’s role in the HD heart phenotype (Cutler et al., 2017). However, hearts in R6/2 mice fail to hypertrophy when exposed to the β-adrenergic agonist isoproterenol (Mielcarek et al., 2014a), and impaired nucleotide metabolism suggests possible mitochondrial dysfunction (Toczek et al., 2016). Hearts in the full-length mHTT transgenic mouse, BACHD, exhibit impaired function, abnormal conduction, increased apoptosis, and fibrosis aggravated by isoproterenol treatment (Schroder et al., 2016). Thus, mHTT expression may impart cardiotoxicity independent from CNS deficits, but mHTT’s specific role in pathogenesis remains unknown.

One of the key regulators of heart size and stress responses is mechanistic Target of Rapamycin complex 1 (mTORC1), a serine-threonine kinase that integrates multiple environmental cues and promotes cellular growth and metabolism (Laplante and Sabatini, 2012). Abolishing mTORC1 activity by ablation of Rheb, the activating subunit of mTORC1, causes defective cardiovascular development (Goorden et al., 2011). Cardiac-specific Rheb knockout (KO) mice have reduced cardiac mRNA translation, retarded cardiomyocyte growth, and impaired cardiac function during the early postnatal period (Tamai et al., 2013; Zhang et al., 2010). mTORC1 activity is less critical for healthy adult hearts, but it plays a key role in mediating the heart’s response to chronic stress. Cardiac-specific raptor deletion, an essential mTORC1 subunit, causes maladaptive responses to pressure overload, resulting in dilated cardiomyopathy and heart failure (Shende et al., 2011). Furthermore, rapamycin, a small molecule mTORC1 inhibitor, prevents cardiac hypertrophy to volume and pressure overload and increases mortality in heart pathology models (Boluyt et al., 1997; Ikeda et al., 2015). Conversely, overexpression of mTORC1’s catalytic subunit, mTOR, attenuates pressure overload-induced fibrosis and reduces inflammatory cytokine responses (Song et al., 2010). Accordingly, mTORC1 dysregulation in HD may explain the decreased heart mass and the increased mortality from heart disease.

Abnormal mTORC1 activity has been studied in HD and may have a critical role in disease pathogenesis. mHTT aggregates sequester mTOR, and inducing autophagy with rapamycin limits aggregates and improves behavioral phenotypes, particularly when administered before symptom onset (Ravikumar et al., 2004). Rapamycin may specifically benefit skeletal muscle in R6/2 mice, a tissue with increased mTORC1 activity (She et al., 2011). However, in other work, mTORC1 activity was notably reduced in HD rodent and human brain, and restoring mTORC1 activity alleviated neuropathology and motor deficits in mouse models of HD (Lee et al., 2015). Given mTORC1’s importance in heart size and stress adaptation, mTORC1 activity may be similarly compromised in the HD heart, causing phenotypic changes that predispose the development of cardiomyopathy.

RESULTS

Decreased Mass in HD Mouse Hearts Results from Limited Growth

We used two HD mouse models to study the effect of HD on the heart. The transgenic N171-82Q mouse expresses the N-terminal 171-amino acid fragment of HTT with an expanded 82 polyglutamine region and causes robust neurological and metabolic phenotypes (Schilling et al., 1999). The zQ175 knockin mouse expresses a human-mouse chimeric mHTT with a 188-residue polyglutamine tract knocked into one of the two endogenous huntingtin alleles (only heterozygotes were used for these studies). zQ175 mice have a slower disease course than the N171-82Q model (Menalled et al., 2012). Importantly, both models express mHTT in the heart.

Heart function in HD mice was assessed by transthoracic echocardiography. N171-82Q mice (18-week-old; end-stage disease) had decreased left ventricular end diastolic (LVEDV) and systolic volumes (LVESV), and decreased heart rate (HR) relative to wild-type (WT) littermates, with a corresponding reduction in cardiac output (CO) (Figure 1A). However, ejection fraction (EF) in transgenic animals was moderately elevated relative to WT (Figure 1A), suggesting HD mice have reduced cardiac mass than their WT littermates but maintain intact ventricular function at end-stage disease.

Excised hearts from N171-82Q mice at end-stage disease were visibly smaller than their WT littermates but with no apparent morphologic differences such as ventricular dilation (Figure 1B). When tracked temporally, decreased heart mass in HD relative to WT mice became apparent at 10 weeks (early-stage disease) and continued to manifest throughout the lifespan (Figure 1C). The growth curves show that the differences between HD and WT heart masses result from failure to gain mass around the time of symptom onset, a trend consistent with the reported pattern of body mass changes in HD patient populations (Hamilton et al., 2004). Mass differences were maintained after normalizing heart mass to tibia length, with the decreased ratio in HD mice indicating uninhibited skeletal growth and ruling out lack of growth factors as an explanation for heart mass differences (Figure 1C). There was no difference in tibia length between WT and N171-82Q animals (Figure S1A). The heart mass-to-body mass ratio does not differ between WT and N171-82Q animals until 14 weeks (mid-stage disease), at which time it is larger in N171-82Q animals relative to WT, suggesting that the heart mass does not change proportionally with the rest of the body (Figure S1B). Indeed, comparing heart mass and body mass in N171-82Q mice over lifespan shows relatively constant heart mass while body mass fluctuates (Figure S1C). In zQ175 animals at 12 months of age (post symptomatic), heart mass and heart mass-to-tibia length are both decreased, similar to and confirming findings in N171-82Q animals (Figure 1D). There were no differences in either tibia length or heart mass to body mass ratio in zQ175 animals (Figures S1D and S1E).

To further address heart mass differences, we used wheat germ agglutinin staining to identify cardiomyocyte borders and measured cross-sectional area. There was no difference in mean cross-sectional area in 6-week-old N171-82Q mice relative to WT littermates, but 14-week-old N171-82Q mice and 12-month-old zQ175 mice had decreased myocardial cross-sectional area relative to WT (Figure 2A). We next used Masson’s trichrome stain to identify fibrosis resulting from cardiomyocyte death but found no evidence of fibrosis in HD hearts from N171-82Q mice at 6 weeks (pre-symptomatic) or 14 weeks of age, or in zQ175 animals at 12 months of age (Figure 2B). Cardiomyocytes isolated from 14-week-old N171-82Q hearts...
(mid-stage disease) had smaller surface area than age-matched WT cardiomyocytes (Figure 2C). TUNEL stain of 18-week N171-82Q hearts showed no evidence of apoptosis (Figure S1 F). Together, these data denote limited cardiomyocyte growth in HD models accounting for mass differences, not cardiomyocyte loss.

Gene signatures associated with heart failure have been detected in HD mouse model hearts (Mielcarek et al., 2014b; Schroeder et al., 2016). Typically, these genes sense stress and limit maladaptive changes. Atrial natriuretic peptide (Anp) and brain natriuretic peptide (Bnp) are hormones expressed in the ventricles and reduce central venous pressure in response to excessive cardiomyocyte stretching (Kinnunen et al., 1993). Anp and Bnp expression were both decreased at 14 and 18 weeks in N171-82Q animals—opposite the expected findings in heart failure—and BNP expression was also decreased at 6 weeks in N171-82Q mice (Figures S1G and S1H). No differences were observed between zQ175 and WT hearts at 12 months. Physiologically significant expression changes in other genes associated with heart failure were not detected in either model (data not shown), confirming functional analyses and suggesting that these mice do not experience heart failure as a result of cardiac mHTT expression.

**mTORC1 Activity Is Decreased in HD Mice Hearts**

We assessed mTORC1 activity in HD mouse model hearts by measuring phosphorylation of the mTORC1 targets ribosomal protein S6 (P-S6) and initiation factor 4E binding protein 1 (P-4EBP1). Both P-S6 and P-4EBP1 levels were decreased relative to WT in N171-82Q hearts beginning at 6 weeks of age and continuing throughout all disease stages; similar decreases in P-S6 and P-4EBP1 were also present in 12-month zQ175 mouse hearts (Figure 3A). Immunohistochemical analysis of P-S6 in N171-82Q hearts at 6 and 14 weeks and zQ175 hearts at
12 months showed P-S6 localizing at what appear to be the intercalated discs between adjacent cardiomyocytes. Consistent with the western blot data, P-S6 stains more intensely in WT hearts than HD hearts (Figure 3B). As a whole, these data indicate decreased mTORC1 activity in HD hearts.

miRNA-Mediated Knock Down of mHTT Restores Cardiac mTORC1 Activity

If mHTT expression causes mTORC1 dysregulation, reducing mHTT levels should ameliorate that phenotype. Cardiac-specific gene transduction was achieved using a previously undescribed cardiotropic variant of AAV2 (CT.AAV) that exhibits robust preference for cardiomyocytes (Figure 4A). 5-week-old, presymptomatic N171-82Q mice were treated with CT.AAV to deliver a miRNA targeting HTT (mi2.1), a non-targeting control (miCTRL), or formulation buffer (FB). Treatment effects were assessed when mice were 16 weeks of age, corresponding to late-stage disease. In mice treated with CT.AAV.mi2.1, qPCR and WB indicated 40% decrease in cardiac HTT mRNA (Figure 4B) and 35% decrease in cardiac HTT protein (Figures 4C and 4D), respectively, with P-S6 and P-4EBP1 levels robustly increased relative to controls (Figures 4E–4G). These results collectively
suggest that cardiac mHTT expression is necessary for mTORC1 inhibition.

**HD Mice Have Blunted Adaptation to Cardiac Stress**

The heart adapts to pathologic stressors (e.g., hypertension, valve stenosis or insufficiencies, myocardial infarction) and maintains cardiac output by hypertrophy, a process mediated by mTORC1. Heart hypertrophy can eventually become maladaptive, but in its early stages it permits continued function and survival. We tested adaptation to cardiac stress in HD using the well-characterized pharmacological model isoprenaline (ISO), a nonspecific β-adrenergic agonist used clinically to increase heart rate, contractility, and conduction speed (Wang et al., 2016). Infusions were started at 12 weeks of age and continued for 14 days; at this age, N171-82Q animals are symptomatic but are not yet experiencing terminal disease. With stress, N171-82Q mice experienced 50% mortality over the course of the study compared to 15% mortality in N171-82Q mice treated with saline and 0% mortality in WT mice treated with either ISO or saline (Figure 5A). Similarly stressed 18-month-old zQ175 mice experienced 83% mortality compared to 25% mortality in stressed WT littermates and 0% in saline-treated mice of either genotype (Figure 5B), confirming that HD mice have increased susceptibility to chronic stress and implicating age as an aggravating factor.

The in vivo cardiac stress response in HD mice was studied by transthoracic echocardiography. Imaging in HD and WT mice was performed at baseline, prior to ISO (or treatment control) delivery, and again 5 and 12 days later, and the temporal change from baseline assessed. ISO-stressed N171-82Q mice had increased fractional shortening (FS), HR, CO, and EF at trial days 5 and 12, but there was no significant change in left ventricular mass (LV mass) (Figures 5C and S2A). Conversely, ISO-stressed WT animals showed significant LV mass increase from baseline at trial days 5 and 12, with unchanged or no significant changes in FS, HR, CO, and EF at both time points (Figures 5C and S2A). ISO did not induce measurable changes in LVEDV or LVESV in either genotype (Figure S2A). These results demonstrate that WT hearts employ a hypertrophic adaptation in response to chronic ISO stress, whereas HD hearts undergo sustained increase of heart rate and contractility but do not hypertrophy.

To confirm limited hypertrophy as measured by echocardiography we measured both gross heart mass and cardiomyocyte cross-sectional area post necropsy and tissue sectioning. Average heart mass/tibia length increased by 35.3% in WT animals treated with ISO relative to saline-treated WT controls and by a blunted 18.7% in HD animals treated with ISO relative to saline-treated HD controls (Figure S2B; note that this data reflect only the surviving mice at trial endpoint). Additionally, ISO induced robust cardiomyocyte cross-sectional area...
increase in WT mice relative to saline-treated controls, but it failed to increase the cross-sectional area of HD cardiomyocytes (Figure 5D). Heart sections stained with Masson's Trichrome (Figure 5E) or picrosirius red (Figure S2C) showed that both WT and HD mice treated with ISO had increased fibrosis relative to saline-treated animals, but the extent of fibrosis was significantly greater in HD hearts. Cardiomyocyte apoptosis in stressed hearts was assessed by TUNEL stain, which was negative for all treatment groups (data not shown). We therefore examined transcripts encoding the apoptosis activator Bax and the anti-apoptotic factor Bcl2. Bax was moderately but significantly elevated in ISO-treated HD and WT hearts relative to saline controls and Bcl2 expression was decreased in stressed hearts (Figure S2D). Taken together, these results demonstrate that HD animals have impaired hypertrophic compensation to cardiac stress with significantly elevated fibrosis.

Decompensated heart failure occurs when pathogenic or compensatory processes become maladaptive and impair cardiac function, which leads to increased pulmonary pressure.
and fluid accumulation in the lungs. The wet/dry lung mass ratio can therefore be used as an indicator of heart failure. In N171-82Q and WT mice treated with ISO or saline for 14 days we observed no differences in the wet/dry lung mass ratio, suggesting that the animals surviving to this time point are not experiencing decompensated heart failure (Figure S2 E). However, only tissue from animals that survived to the end of the trial were included in the analysis due to rapid, confounding, post-mortem lung changes. Additionally, in stressed hearts from both WT and HD animals that survived to trial endpoint, mTORC1 activity was increased as measured by P-S6/S6 levels, with significant variability in HD hearts (Figure S2 F). P-4EBP1/4EBP1 levels were not different among treatment groups, with a similar large variance observed in surviving stressed HD mice hearts (Figure 5 E). These observations suggest that partially overcoming the inherent cardiac mTORC1 dysregulation in HD mice may be associated with survival to trial endpoint.

mTORC1 Activation in HD Hearts Restores the Adaptive Cardiac Stress Response

We studied the relevance of mTORC1 inhibition in the HD cardiac stress response by selectively activating cardiac mTORC1 in N171-82Q hearts. CT.AAV was used to express a constitutively active variant of Rheb (Rheb-S16H, or simply caRheb) or GFP in HD mice hearts (Yan et al., 2006). Successful caRheb transduction was confirmed by western blot (Figure S3 A). Two weeks after vector delivery, heart stress was induced by ISO or saline infusion as before (Figure 6 A). As expected, HD mice transduced with CT.AAV.GFP and treated with ISO (GFP-ISO) experienced increased mortality relative to saline-treated mice transduced with either CT.AAV.GFP or CT.AAV.caRheb (GFP-saline and caRheb-saline, respectively) (Figure 6 B). In contrast, HD mice transduced with CT.AAV.caRheb and treated with isoprenaline (caRheb-ISO), displayed significantly less mortality over the course of the study (Figure 6 B). Thus, cardiac caRheb expression ameliorates sensitivity to ISO stress in HD mice.

On transthoracic echocardiogram, the GFP-ISO group displayed increased FS, HR, and EF from baseline without an increase in LV mass at trial days 5 and 12 (Figures 6 C and S3B), similar to the observations made in stressed non-AAV-treated N171-82Q hearts (Figures 5 C and S2 A). Conversely,
the caRheb-ISO group responded to stress with an increase in LV size from pretreatment levels, with no changes in FS, HR, or EF from pretreatment levels at either time point (Figures 6C and S3B), approximating the WT response to chronic ISO (Figures 5C and S2A). There were no measurable changes in LVEDV, LVESV, and CO for either vector (Figure S3B). These measurements collectively imply that caRheb transduction restores the functional stress response in HD hearts.

The effects of caRheb transduction on the cardiac stress response in HD mice were further studied by examining heart mass/tibia length. There were no differences observed between GFP-saline, GFP-ISO, and caRheb-saline groups. However, caRheb-ISO-treated HD mice showed a 27.5% increase in heart mass/tibia length (Figure S3C). Analysis of cardiomyocyte cross-sectional area in the HD mice showed no difference between the GFP-saline and GFP-ISO groups (Figure 6D). In caRheb-treated HD mice, the saline group had moderately increased cardiomyocyte cross-sectional area relative to the GFP-saline group. Notably, caRheb-ISO-treated mice showed robust cross-sectional area increase relative to all other groups, representing restored hypertrophic adaptation (Figure 6D). The impact of caRheb on restoration of proper compensation to chronic stress was further apparent in analyses of fibrosis by Masson’s trichrome (Figure 6E) and picrosirius red stains (Figure S3D): extensive fibrosis was present in hearts from the GFP-ISO-treated HD mice, whereas fibrosis in hearts from the caRheb-ISO group was almost entirely absent (Figure 6E). TUNEL stain to detect apoptotic nuclei was negative in all treatment groups (data not shown), but qPCR analysis of apoptotic factors showed moderately but significantly increased Bax expression in the caRheb-ISO group only, and no significant changes in Bcl2 expression (Figure S3E). Together, these results show that caRheb expression in HD hearts allows cardiomyocyte hypertrophy and prevents deposition of fibrotic tissue in response to stress.

Similar to before, wet/dry lung mass ratios of mice surviving to trial endpoint were not different between groups, suggesting that the mice included in the assay were not experiencing decompensated heart failure at the time of observation (Figure S3F). Additionally, as before, mTORC1 activity in hearts of surviving...
Figure 7. Analysis of Upstream mTORC1 Pathway Signaling in HD Mouse Hearts

(A) Simplified schematic of mTORC1 activation pathway. Arrowheads represent activation step; blunted lines indicate inhibitory step. Some steps are not explicitly listed and are represented by multiple arrows.

(B) IGF-I levels in mouse serum from N171-82Q mice at 6 weeks (n ≥ 5) and 14 weeks (n ≥ 7) of age and from zQ175 mice at 6 months (n ≥ 5) and 12 months of age (n ≥ 12). Data are expressed relative to WT for each time point.

(C) Igf1r expression measured by qPCR in N171-82Q mice at 6 (n ≥ 6), 10 (n ≥ 7), 14 (n ≥ 8), and 18 (n ≥ 8) weeks of age, and in zQ175 mice at 12 months of age (n ≥ 10). Igf1r expression levels were normalized to Tbp expression as an internal control and are graphed relative to WT levels.

(D) Pten expression in N171-82Q mice at 6 (n ≥ 6), 10 (n ≥ 7), 14 (n ≥ 8), and 18 (n ≥ 8) weeks of age, and in zQ175 mice at 12 months of age (n ≥ 10), measured by qPCR. Gene expression levels were normalized to expression of Tbp mRNA as an internal control and are graphed relative to WT levels.

(legend continued on next page)
animals was increased with ISO treatment (Figure S3G). While P-S6/S6 levels in caRheb-transduced mice groups trended higher than their respective GFP-transduced mice groups, they did not achieve statistical significance due in part to large variance in surviving GFP-ISO samples. Thus, the ability to overcome the dysregulated cardiac mTORC1 activity in HD hearts, through endogenous or exogenous means, is associated with survival in response to stress.

**Molecular Mechanism of Cardiac Dysregulation in HD**
We analyzed upstream members in the mTORC1 pathway to determine the molecular basis for mTORC1 dysregulation in HD mice (Figure 7A). We first asked if decreased cardiac mTORC1 activity is due to decreased axis activation. IGF-I, a P38-Akt-mTORC1 pathway activator, was unexpectedly increased in 6-week-old N171-82Q mice serum relative to WT, but normalized by 14 weeks (Figure 7B). Serum IGF-I was unchanged in presymptomatic 6-month zQ175 mice and slightly decreased in 12-month zQ175. Expression of IGF-I receptor RNA, Igf1r, was equal between N171-82Q and WT hearts at 6 weeks and between zQ175 and WT hearts at 12 months, but increased in N171-82Q hearts relative to WT at later time points (Figure 7C). Phosphorylation of Erk, a downstream target of IGF-I signaling independent of the mTORC1 pathway, did not differ between HD and WT at any time point in N171-82Q mice or in 12-month-old zQ175 mice (Figure S4A). Insulin, another mTORC1 pathway activator, was decreased in 14-week N171-82Q serum (Figure S4B). Note that N171-82Q mice at this stage experience altered feeding patterns (Figure S4C) that could affect insulin secretion.

Next, we examined intracellular pathways that regulate mTORC1. Modest differences existed in phosphatase and tensin homolog (Pten) mRNA expression at 6 weeks, an early negative regulator of the mTORC1 pathway, but the changes were so slight that functional significance is unlikely. No differences existed at later time points or in 12-month zQ175 animals (Figure 7D). Growth factor signals are transmitted intracellularly to mTORC1 via the kinases PDK1 and Akt. Phosphorylated Akt (P-Akt, indicative of PDK1 activity) and phosphorylated mTOR (P-mTOR, indicative of Akt activity) were both significantly decreased in 6-week N171-82Q hearts relative to WT and in 12-month zQ175 hearts (Figures 7E–7G). However, P-Akt and P-mTOR levels were not different between HD and WT at post-symptomatic time points in N171-82Q mice. The early decrease, followed by normalization, suggests that the diminished PDK1 activity impairs mTORC1 activation at presymptomatic and early disease stages, but not later stages.

We next examined phosphorylation of AMPKα, which occurs in response to energy deficits within cells and permits AMP kinase (AMPK) to inhibit mTORC1. P-AMPKα levels were not different at any time point in N171-82Q mice or in 12-month zQ175 mice relative to WT mice (Figure 7E and H), suggesting that the noted cardiac mTORC1 dysregulation does not result from systemic energy imbalance.

The forkhead box protein O (FoxO) transcription factor family coordinates expression of genes that generally oppose the downstream functions of mTORC1 (Puthanveetil et al., 2013). Gene targets of the FoxO family include genes products that contribute to muscle atrophy, as well as Bnip3, an inhibitor of mTORC1 (Li et al., 2007; Lin et al., 2014). Previous reports have associated HD with increased FoxO3 expression in the brain (Kannike et al., 2014). FoxOT expression was elevated relative to WT in both in N171-82Q hearts starting at 6 weeks and in zQ175 mouse hearts at 12 months (Figure S5A). FoxO3 expression was elevated only in N171-82Q hearts at 14 and 18 weeks (Figure S5B). Expression of Bnip3 in HD hearts did not differ from WT at any time point in N171-82Q mice or in zQ175 hearts (Figure S5C). These data suggest that the FoxO pathway is not impairing mTORC1 activity in the heart.

Activation of mTORC1 relies both on signaling cascades and on proper localization of the various subunits of the complex to internal membranes. As none of the probable signaling pathways could explain the mTORC1 dysregulation in either model, we examined subcellular localization of mTORC1 components. Membrane extraction on N171-82Q mouse hearts at 16 weeks of age showed that Rheb, the activating subunit of mTORC1, was increased in the cytoplasmic fraction and decreased in the membrane fraction (Figure 7I). Rheb localization did not differ between N171-82Q and WT hearts at 6 weeks of age (Figure S6A), consistent with previous results showing differences in mTORC1 dysregulation between presymptomatic and symptomatic HD mice. Altering Rheb’s ability to properly localize has previously been shown to inhibit mTORC1 activity, and thus Rheb mislocalization may underlie the impairment of mTORC1 in symptomatic HD mouse hearts. Additionally, caRheb overexpression could overcome the deficiency imparted by endogenous mislocalization.

**DISCUSSION**
HD is a neurodegenerative disease, but ubiquitous mHTT expression has adverse effects outside of the CNS. Indeed, epidemiological data indicate that the heart plays a central role as a major component of mortality in HD patients (Lanska et al., 1988a, 1988b; Sørensen and Fenger, 1992). Here, we characterized the cardiac phenotype resulting from mHTT expression in two HD mouse models and found that HD hearts have decreased size but are not functionally impaired in the absence of cardiac stress. Additionally, cardiac mTORC1 is dysregulated in HD murine models, and the knock down of mHTT

(E) Representative blots of N171-82Q mice at 6 (n ≥ 6), 10 (n ≥ 7), 14 (n ≥ 8), and 18 (n ≥ 8) weeks of age, and from zQ175 mice at 12 months of age (n ≥ 10).
(F–H) Graphs for (F) P-Akt/Akt, (G) P-mTOR/mTOR, and (H) P-AMPKα/AMPKα represent quantitation of band densities for phospho-specific antibodies normalized to band densities of phospho-independent antibodies and are expressed relative to WT levels.
(I) Representative western blot and quantitation from membrane purification of 16 week-old N171-82Q hearts. Loading controls were α-tubulin for cytoplasmic fractions, Na/K-ATPase for membrane fractions. For each group n = 6. Graphs represent Rheb density normalized to loading control from the corresponding lane and are expressed relative to WT.
For all panels, Student’s t test, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Error bars represent SEM. See also Figures S4–S6.
restores mTORC1 activity. The physiological relevance of the mTORC1 dysregulation in the HD heart is that it prevents proper adaptation to hemodynamic stress and sensitizes the heart to pathologic conditions; under cardiovascular stress conditions HD mice exhibit increased mortality, blunted heart hypertrophy, and extensive myocardial fibrosis. Importantly, these abnormalities were abrogated following activation of cardiac mTORC1, implicating decreased mTORC1 activity as central to development of heart disease with stress in HD mice. In the transgenic and KI models studied, chronic mTORC1 dysregulation in HD is not explained by inhibitory upstream signaling. However, Rheb’s subcellular localization is altered, which can negatively impact mTORC1 activity (Basso et al., 2005; Buerger et al., 2006; Castro et al., 2003). Cumulatively our data suggest that mHTT’s impact on mTORC1 impairs the ability of cardiomyocytes to respond to stressors, which may underlie the etiology of heart disease-induced death in HD patients.

A recent review on peripheral HD phenotypes presents data that identifies HTT mRNA expression within the heart but suggests no HTT protein expression (Carroll et al., 2015). This conclusion is based on data from the Human Protein Atlas, which uses a qualitative IHC approach and assigns a score of zero if at least one out of two antibodies fails to produce a signal; thus, lack of expression and uncertain assay results are considered equivalent (Uhlen et al., 2015). Fortunately, multiple high-throughput proteomics analyses using mass spectrometry have identified HTT protein within the heart, consistent with the RNA sequencing (RNA-seq) data (Wang et al., 2012; Wilhelm et al., 2014).

Functional analyses in cardiac stress experiments showed that HD hearts experience increased rate and contractility in response to chronic isoproterenol, whereas WT hearts (and HD hearts transduced with CT.AAV.caRheb) respond by hypertrophy. Heart hypertrophy is frequently considered a pathologic process, and indeed, substantial proliferation and reorganization of cardiac myofibrils can decrease efficiency and conduction, restrict blood supply, and lead to heart failure (Beache et al., 2001; Norton et al., 2002). However, before the compensatory processes become pathologic in and of themselves, hypertrophy decreases the amount of work the heart must exert in response to a stressor (according to the Laplace law) and can therefore be considered an indicator of an underlying pathology before it becomes maladaptive (Cotecchia et al., 2015). Consequently, without hypertrophy, the HD hearts’ response to stress requires more work—therefore energy—than their WT littermates. This chronic, high-energy response, coupled with functional mitochondrial abnormalities (Toczek et al., 2016), may make HD cardiomyocytes more susceptible to exhaustion and might explain the increased mortality and fibrosis observed in stressed HD animals. While characterizing the specific pathophysiology of stressed HD hearts is outside the scope of these studies, future efforts to understand mortality in stressed HD mouse hearts could employ ex vivo bioenergetics to confirm unbalanced ATP metabolism, electrocardiographic telemetry to determine if fatal arrhythmias contribute to death, and invasive hemodynamics to assess pressure, resistance, and flow changes upon chronic stress.

Similar to studies in brain (Lee et al., 2015), cardiac mTORC1 activity was dysregulated before motor symptom onset, suggesting that molecular pathogenesis associated with mHTT expression begins early and contributes to the notable, limited heart growth. Interestingly, the cause of mTORC1 dysregulation results from distinct etiologies based on disease stage. Presymptomatic mTORC1 dysregulation is associated with decreased upstream activation, whereas upstream signaling activity is normal in post-symptomatic HD mice. The later-stage mTORC1 dysregulation, even when upstream signals are normal, is likely due to Rheb mislocalization. Rheb is post-translationally modified with a farnesyl group that targets it to the cellular endomembranes in the ER, Golgi, mitochondria, and late endosomes/lysosomes (Buerger et al., 2006; Sancak et al., 2008). Inhibiting this process induces Rheb mislocalization and impairs mTORC1 activity (Basso et al., 2005; Castro et al., 2003). Farnesyl is a metabolic precursor product in the cholesterol biosynthesis pathway, a process impaired in HD (Leoni and Caccia, 2015), and lysosomal cholesterol stores are essential for proper mTORC1 localization and activation (Castellano et al., 2017). While it is still unknown if Rheb in late-stage HD hearts mislocalizes due to lack of farnesylation or through another mechanism, it is possible that aberrant cholesterol metabolism in HD may contribute to the decreased mTORC1 activity observed in both the heart and the brain.

With CNS-directed HTT-lowering therapy in HD patients progressing in clinical trials (Ionis Pharmaceuticals, 2017), there is a pressing need to identify the consequences of mHTT expression in the heart and other peripheral organs that may be unmasked with amelioration of CNS deficits. Future studies on human HD heart tissues, which at the present time are not routinely collected, will help illuminate the temporal nature of reduced cardiac mass in HD patients, and if mTORC1 plays as central a role in the phenotype, Our data are also relevant to those contemplating the use of mTORC1 inhibitors in HD, raising cardiac issues as a possible contraindication. Finally, this work demonstrates the pressing need within the field for expanded tissue banks and clinical studies to adequately understand the heart and other peripheral phenotypes in HD, as well as other neurodegenerative diseases.

**EXPERIMENTAL PROCEDURES**

**Study Approval**

All animal protocols were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee at either the University of Iowa or The Children’s Hospital of Philadelphia.

**Animals**

N171-82Q and zQ175 HD murine model lines were obtained from Jackson Laboratories and maintained on a B6C3F1/J (N171-82Q) or C56B6/J (zQ175) background. Mice were genotyped using primers specific for human HTT, and age-matched WT littermates were used as controls for all experiments. Animals were housed in enriched, temperature-controlled environments with a 12-hr light/dark cycle. Food and water were provided ad libitum.

**Echocardiography**

Mice were anesthetized with 2.5% isoflurane and positioned on a heated stage with electrocardiogram (EKG) measurement. Transthoracic echocardiograms were recorded using an MS-400 30 MHz probe connected to a Vevo 2100 or 3100 imager with cardiovascular measurement package. (Fujifilm VisualSonics). Images were acquired and dimensions measured by an experienced
operator blinded to mouse genotype. Cardiac output was calculated as the difference between left ventricular end diastolic and systolic volumes multiplied by the heart rate.

**Histology Analyses**

For wheat germ agglutinin staining, images were taken throughout the posterior wall to encompass all transversely sectioned cells. ImageJ was used to overlay a grid on each image to randomly select approximately 50 cells/image and cross-sectional area was measured for each selected cell. At least 200 cells were measured per heart. For picrosirius red-stained sections, low-power images were taken using both a standard bright field filter and a polarizing filter. At least 9 images were collected per heart, capturing a large section of the left ventricle. Large vessels were avoided because of their high collagen content. ImageJ was used to threshold and measure area of each image, and percent of total area occupied by fibrotic tissue was used for analyses.

**Western Blot Analysis**

Densitometry was performed using NIH ImageJ software. Densities of bands detected by phospho-antibodies were normalized to bands detected by phospho-independent antibodies in same lane. Phospho-independent antibodies were normalized to α-tubulin or Na/K-ATPase band in same lane.

**IGF-I and Insulin Measurements**

Mouse blood was collected retro-orbitally using microcapillary tubes. Blood was allowed to clot for 15 min at room temperature, and serum was isolated by centrifugation. Serum IGF-I and insulin was detected by colorimetric ELISA (IGF-I, Thermo Fisher Scientific; insulin, Crystal Chem).

**qPCR Analyses**

Relative gene expression was determined using the ΔΔCT method, normalizing to TATA-binding protein (TBP) or Gapdh. Standard curves to determine copies/µg RNA were prepared using plasmids containing the gene of interest.

**Plasmids and AAVs**

Cardiotropic AAV (termed CT.AAV) was developed by inserting a nine-residue peptide sequence identified by phage panning into the cap gene of AAV2. Genes of interest were cloned into a standard AAV2 expression cassette. MicroRNA systems (mi21, miCTRL) were previously developed by the lab of Bhrar et al. (2008) and driven by a U6 promoter. Rheb-S169 (constitively active Rheb, caRheb) plasmids were developed as previously described and were driven by a CAG promoter (CMV enhancer linked to a β-actin promoter) (Lee et al., 2015). All AAVs were made by the Research Vector Core at The Children's Hospital of Philadelphia. Titers (measured in viral genomes/mL) were determined by qPCR.

**Cardiac Stress Induction**

Isoproterenol (ISO, Sigma) was dissolved in sterile PBS and administered by continuous infusion using implantable mini-osmotic pumps (Alzet) at a dose of 30 mg/kg body mass/day. Pumps were set to deliver and primed in a sterile environment and were inserted into the dorsal subcutaneous space caudal to the scapulae.

**Statistics**

Data were analyzed with Student’s t test or one-way ANOVA with Tukey’s post hoc analysis to assess differences in experimental groups. All statistical tests were two-sided. Normality was determined graphically and by Shapiro-Wilk test. Survival curves were analyzed by Mantel-Cox (log-rank) test. All analyses were performed using GraphPad Prism version 7.03 or R version 3.2.2. Data are expressed as mean ± SEM, and for all analyses, p ≤ 0.05 was considered significant.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.03.117.

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**AUTHOR CONTRIBUTIONS**

D.D.C., J.H.L., and B.L.D. designed the studies. D.D.C., C.J.P., and J.H.L. performed the experiments, collected the data, and analyzed the results. D.D.C., J.H.L., and A.M.M. designed and cloned the plasmids. Y.H.C. designed and cloned the CT.AAV construct. D.D.C. and B.L.D. wrote the manuscript.

**DECLARATION OF INTERESTS**

B.L.D. is a founder of Spark Therapeutics and is on the SAB of Intellia Therapeutics and Sarepta Therapeutics. None of this work was sponsored by, or reagents developed or licensed to, any of these entities at the time of submission.

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**REFERENCES**

Ablidstrup, M., and Shattock, M. (2013). Cardiac dysautonomia in Huntington’s disease. J. Huntington’s Dis. 2, 251–261.

Bär, K.J., Boettger, M.K., Andrich, J., Epplen, J.T., Fischer, F., Cordes, J., Koschke, M., and Age-link, M.W. (2008). Cardiovascular modulation upon postural change is altered in Huntington’s disease. Eur. J. Neurol. 15, 869–871.

Basso, A.D., Mirza, A., Liu, G., Long, B.J., Bishop, W.R., and Kirschmeier, P. (2005). The farnesyl transferase inhibitor (FTI) SCH66336 (lonafarnib) inhibits RhoB farnesylation and mTOR signaling. Role in FTI enhancement of taxane and tamoxifen anti-tumor activity. J. Biol. Chem. 280, 31101–31108.

Beache, G.M., Herzka, D.A., Boxerman, J.L., Post, W.S., Gupta, S.N., Faranesh, A.Z., Solaiyappan, M., Bottomley, P.A., Weiss, J.L., Shapiro, E.P., and Hill, M.N. (2001). Attenuated myocardial vasodilator response in patients with hypertensive hypertrophy revealed by oxygenation-dependent magnetic resonance imaging. Circulation 104, 1214–1217.

Bellosa Diago, E., Pérez-Pérez, J., Santos Lasaosa, S., Víctoria Álebesque, A., Martínez-Horta, S., Kulisevsky, J., and López Del Val, J. (2018). Neurocardiovascular pathology in Premanifest and Early Stage Huntington’s Disease. Eur. J. Neurol. Published online March 14, 2018. https://doi.org/10.1111/ene.13630.

Boluyt, M.O., Zheng, J.S., Younes, A., Long, X., O’Neill, L., Silverman, H., La-katta, E.G., and Crow, M.T. (1997). Rapamycin inhibits alpha 1-adrenergic receptor-stimulated cardiac myocyte hypertrophy but not activation of hypertrophy-associated genes. Evidence for involvement of p70 S6 kinase. Circ. Res. 81, 176–186.

Boudreau, R.L., McBride, J.L., Martins, I., Shen, S., Xing, Y., Carter, B.J., and Davidson, B.L. (2009). Nonallelic-specific silencing of mutant and wild-type huntingtin demonstrates therapeutic efficacy in Huntington’s disease mice. Mol. Ther. 17, 1053–1063.

Buerger, C., DeVries, B., and Stambolic, V. (2006). Localization of Rheb to the endomembrane is critical for its signaling function. Biochem. Biophys. Res. Commun. 344, 869–880.

Carroll, J.B., Bates, G.P., Steffan, J., Saft, C., and Tabrizi, S.J. (2015). Treating huntingtin demonstrates therapeutic efficacy in Huntington’s disease. Lancet Neurol. 14, 1135–1142.
Nishizaki, A., and Sunagawa, K. (2015). The Akt-mTOR axis is a pivotal regulator of Huntington’s disease. J. Neurol. Neurosurg. Psychiatry, 32493–32496.

CDC. (1994). National Health and Nutrition Examination Survey: NHANES III (1988-1994). https://wwwn.cdc.gov/nchs/nhanes/nhanes3/default.aspx.

Chaturvedi, R.K., Caliganas, N.Y., Yang, L., Hennessey, T., Johri, A., and Beal, M.F. (2010). Impairment of PGC-1alpha expression, neuropathology and hepatic steatosis in a transgenic mouse model of Huntington’s disease following chronic energy deprivation. Hum. Mol. Genet. 19, 3190–3205.

Castecia, S., Del Vescovo, C.D., Colella, M., Caso, S., and Diviani, D. (2015). The alpha-1-adrenergic receptors in cardiac hypertrophy: signaling mechanisms and functional implications. Cell. Signal. 27, 1984–1993.

Cutler, T.S., Park, S., Loh, D.H., Jordan, M.C., Yokota, T., Roos, K.P., Ghiani, C.A., and Colwell, C.S. (2017). Neurocardiovascular deficits in the Q175 mouse model of Huntington’s disease. Physiol. Rep. 5, e13289.

Goorden, S.M., Hoogeveen-Westerveld, M., Cheng, C., van Woerden, G.M., Mozaffari, M., Post, L., Duckers, H.J., Nellist, M., and Elgersma, Y. (2011). Rheb is essential for murein development. Mol. Cell. Biol. 31, 1672–1678.

Hamilton, J.M., Wolfson, T., Peavy, G.M., Jacobson, M.W., and Corey-Bloom, J.; Huntington Study Group (2004). Rate and correlates of weight change in Huntington’s disease. J. Neurol. Neurosurg. Psychiatry 75, 209–212.

Harper, S.Q., Staber, P.D., He, X., Eliason, S.L., Martins, I.H., Mao, Q., Yang, L., Kotin, R.M., Paulson, H.L., and Davidson, B.L. (2005). RNA interference exerts a unique regulation of mTORC1 and cell survival under energy stress. Oncogene 33, 3183–3194.

McBride, J.L., Pitzer, M.R., Boudreau, R.L., Dufour, B., Hobbs, T., Ojeda, S.R., and Davidson, B.L. (2011). Preclinical safety of RNAi-mediated HTT suppression in the rhesus macaque as a potential therapy for Huntington’s disease. Mol. Ther. 19, 2152–2162.

Meikami, G.C., Trujillo, A.S., Ramos, R., Bodmer, R., Bernstein, S.I., and Ocor, K. (2013). Huntington’s disease induced cardiac amyloidosis is reversed by modulating protein folding and oxidative stress pathways in the Drosophila heart. PLoS Genet. 9, e1004024.

Mennaji, L.B., Kudwa, A.E., Miller, S., Fitzpatrick, J., Watson-Johnson, J., Keating, N., Ruiz, M., Mushlin, R., Alosio, W., McConnell, K., et al. (2012). Comprehensive behavioral and molecular characterization of a new knock-in mouse model of Huntington’s disease: zQ75. PLoS ONE 7, e49838.

Mielcarek, M., Bondulich, M.K., Inuabasi, L., Franklin, S.A., Muller, T., and Bates, G.P. (2014a). The Huntington’s disease-related cardiomyopathy prevents a hypertrophic response in the R6/2 mouse model. PLoS ONE 9, e108961.

Mielcarek, M., Inuabasi, L., Bondulich, M.K., Muller, T., Osborne, G.F., Franklin, S.A., Smith, D.L., Neudeker, A., Rosinski, J., Rattray, I., et al. (2014b). Dysfunction of the CNS-heart axis in mouse models of Huntington’s disease. PLoS Genet. 10, e1004550.

Mihm, M.J., Amann, D.M., Schanbacher, B.L., Altschuld, R.A., Bauer, J.A., and Hoyt, K.R. (2007). Cardiac dysfunction in the R6/2 mouse model of Huntington’s disease. Neurobiol. Dis. 25, 297–308.

Myers, R.H., Marans, K., and MacDonald, M.E. (1998). Huntington’s Disease. In Genetic Instabilities and Hereditary Neurological Diseases, S.T. Warren and R.T. Wells, eds. (The University of Michigan: Academic Press), pp. 301–323.

Norton, G.R., Woodwiss, A.J., Gaasch, W.H., Mela, T., Chung, E.S., Auriemma, G.P., and Meyer, T.E. (2002). Heart failure in pressure overload hypertrophy. The relative roles of ventricular remodeling and myocardial dysfunction. J. Am. Coll. Cardiol. 39, 666–671.

Pattison, J.S., Sanbe, A., Maloyan, A., Osinska, H., Kleivitsky, R., and Robbins, J. (2008). Cardiomyocyte expression of a polyclutamine premyloid oligomer causes heart failure. Circulation 117, 2743–2751.

Puthanveetil, P., Wan, A., and Rodrigues, B. (2013). FoxO1 is crucial for sustaining cardiomyocyte metabolism and cell survival. Cardiovasc. Res. 97, 393–403.

Ravikumar, B., Vacher, C., Berger, Z., Davies, J.E., Luo, S., Oroz, L.Z., Scaravilli, F., Easton, D.F., Duden, R., O’Kane, C.J., and Rubinstein, D.C. (2004). Inhibition of mTOR induces autophagy and reduces toxicity of polyclutamine expansions in fly and mouse models of Huntington disease. Nat. Genet. 36, 585–595.

Ribchester, R.R., Thomson, D., Wood, N.L., Hinks, T., Gillingwater, T.H., Wishart, T.M., Court, F.A., and Morton, A.J. (2004). Progressive abnormalities in skeletal muscle and neuromuscular junctions of transgenic mice expressing the Huntington’s disease mutation. Eur. J. Neurosci. 20, 3092–3114.

Rosas, H.D., Goodman, J., Chen, Y.I., Jenkins, B.G., Kennedy, D.N., Makris, N., Patti, M., Seidman, J.L., Beal, M.F., and Koroshetz, W.J. (2001). Striatal volume loss in HD as measured by MRI and the influence of CAG repeat frequency. Neurology 57, 1025–1028.

Sancak, Y., Peterson, T.R., Shaul, Y.D., Lindquist, R.A., Thoren, C.C., Bar-Peled, L., and Sabatini, D.M. (2008). The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. Science 320, 1496–1501.

Schilling, G., Becher, M.W., Sharp, A.H., Jinnah, H.A., Duan, K., Kotzuk, J.A., Slunt, H.H., Ratovitski, T., Cooper, J.K., Jenkins, N.A., et al. (1999). Intracellular inclusions and neuritic aggregates in transgenic mice expressing a mutant N-terminal fragment of huntingtin. Hum. Mol. Genet. 8, 397–407.
Schroeder, A.M., Wang, H.B., Park, S., Jordan, M.C., Gao, F., Coppola, G., Fishbein, M.C., Roos, K.P., Ghiante, C.A., and Colwell, C.S. (2016). Cardiac dysfunction in the BACHD mouse model of Huntington’s disease. PLoS ONE 11, e0147269.

Sharma, K.R., Romano, J.G., Ayar, D.R., Rotta, F.T., Facca, A., and Sanchez-Ramos, J. (1999). Symptomatic skin response and heart rate variability in patients with Huntington disease. Arch. Neurol. 56, 1248–1252.

She, P., Zhang, Z., Marchionini, D., Diaz, W.C., Jetton, T.J., Kimball, S.R., Vary, T.C., Lang, C.H., and Lynch, C.J. (2011). Molecular characterization of skeletal muscle atrophy in the R6/2 mouse model of Huntington’s disease. Am. J. Physiol. Endocrinol. Metab. 307, E49–E61.

Shende, P., Piasance, I., Morandi, C., Pellieux, C., Berthonneche, C., Zorzato, F., Krishnan, J., Lerch, R., Hall, M.N., Ruegg, M.A., et al. (2011). Cardiac raptor ablation impairs adaptive hypertrophy, alters metabolic gene expression, and causes heart failure in mice. Circulation 123, 1073–1082.

Song, X., Kusakari, Y., Xiao, C.Y., Kinsella, S.D., Rosenberg, M.A., Scherrer-Crosbie, M., Hara, K., Rosenzweig, A., and Matsu, T. (2010). mTOR attenuates the inflammatory response in cardiomyocytes and prevents cardiac dysfunction in pathological hypertrophy. Am. J. Physiol. Cell Physiol. 299, C1256–C1266.

Sørensen, S.A., and Fenger, K. (1992). Causes of death in patients with Huntington’s disease and in unaffected first degree relatives. J. Med. Genet. 29, 911–914.

Stanek, L.M., Yang, W., Angus, S., Sardi, P.S., Hayden, M.R., Hung, G.H., Bennett, C.F., Cheng, S.H., and Shihabuddin, L.S. (2013). Antisense oligonucleotide-mediated correction of transcriptional dysregulation is correlated with behavioral benefits in the YAC128 mouse model of Huntington’s disease. J. Huntington’s Dis. 2, 217–228.

Tamai, T., Yamaguchi, O., Hikos, S., Takeda, T., Taneike, M., Oka, T., Oyabu, J., Murakawa, T., Nakayama, H., Uno, Y., et al. (2013). Rheb (Ras homologue enriched in brain)-dependent mammalian target of rapamycin complex 1 (mTORC1) activation becomes indispensable for cardiac hypertrophic growth after early postnatal period. J. Biol. Chem. 288, 10176–10187.

The Huntington’s Disease Collaborative Research Group (1993). A novel gene containing a trinucleotide repeat that is expanded and unstable on Hunting-}

Trotter, Y., Devys, D., Imbert, G., Saudou, F., An, I., Lutz, Y., Weber, C., Agid, Y., Hirsch, E.C., and Mandel, J.L. (1995). Cellular localization of the Huntington’s disease protein and discrimination of the normal and mutated form. Nat. Genet. 10, 104–110.

Uhlén, M., Fagerberg, L., Hallström, B.M., Lindskog, C., Oksvold, P., Mardinoglu, A., Sivertsson, Å., Kampf, C., Sjöstedt, E., Asplund, A., et al. (2015). Proteomics. Tissue-based map of the human proteome. Science 347, 1260419.

Walker, F.O. (2007). Huntington’s disease. Lancet 369, 218–228.

Wang, M., Weiss, M., Simonovic, M., Haertinger, G., Schrimer, S.P., Hengartner, M.O., and von Mering, C. (2012). PaxDb, a database of protein abundance averages across all three domains of life. Mol. Cell. Proteomics 11, 492–500.

Wang, J.J., Rau, C., Avetisyan, R., Ren, S., Romay, M.C., Stolín, G., Gong, K.W., Wang, Y., and Lusis, A.J. (2016). Genetic dissection of cardiac remodeling in an isoproterenol-induced heart failure mouse model. PLoS Genet. 12, e1006038.

Weixel, N.S., Lorimer, J., Porter, J., Gomez, F., Moskowitz, C., Shackell, E., Marder, K., Penchaszadeh, G., Roberts, S.A., Mayán, J., et al.; U.S.-Venezuela Collaborative Research Project (2004). Venezuelan kindreds reveal that genetic and environmental factors modulate Huntington’s disease age of onset. Proc. Natl. Acad. Sci. USA 101, 3498–3503.

Wilhelm, M., Schlegl, J., Hahne, H., Gholami, A.M., Lieberenz, M., Savitski, M.M., Ziegler, E., Butzmann, L., Gessulat, S., Marx, H., et al. (2014). Mass-spectrometry-based draft of the human proteome. Nature 509, 582–587.

Wood, N.I., Sawiak, S.J., Buonincontri, G., Niu, X., Kane, A.D., Carpenter, T.A., Giussani, D.A., and Morton, A.J. (2012). Direct evidence of progressive cardiac dysfunction in a transgenic mouse model of Huntington’s disease. J. Huntington’s Dis. 1, 57–64.

Yan, L., Findlay, G.M., Jones, R., Procter, J., Cao, Y., and Lamb, R.F. (2006). Hyperactivation of mammalian target of rapamycin (mTOR) signaling by a gain-of-function mutant of the Rheb GTPase. J. Biol. Chem. 281, 19793–19797.

Zhang, D., Contu, R., Latronico, M.V., Zhang, J., Rizzi, R., Catalucci, D., Miyamoto, S., Huang, K., Ceci, M., Gu, Y., et al. (2010). MTORC1 regulates cardiac function and myocyte survival through 4E-BP1 inhibition in mice. J. Clin. Invest. 120, 2805–2816.
Supplemental Information

Cardiac mTORC1 Dysregulation Impacts Stress Adaptation and Survival in Huntington's Disease

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Supplemental Experimental Procedures

Heart Mass Analysis: Mice were anesthetized with a mixture of ketamine/xyline (IP injection of 100 mg/kg and 10 mg/kg, respectively) and transcardially perfused with cold saline. Whole hearts were excised, blot-dried, and massed on an analytical balance. Left tibias were isolated and measured with electronic calipers.

Histology: Excised hearts were bisected transversely at the approximate midpoint and apical portions were post-fixed overnight in 4% paraformaldehyde. Heart tissue was dehydrated, embedded in paraffin, and sectioned to 5 µm on a Microm HM 355s microtome. Mounted sections were heated at 55°C for 20 minutes and rehydrated. The following protocols were then undertaken:

- Wheat germ agglutinin: slides were incubated in a 10 µg/ml solution of wheat germ agglutinin conjugated either to Alexa-488 or Alexa-594 (Thermo Fisher) and 2 µg/ml Hoechst stain for 15 minutes at 37°C.
- Masson’s trichrome stain: slides were incubated in 56°C Bouin’s solution (Sigma) for 10 minutes, followed by staining with Masson’s Trichrome (Sigma) according to manufacturer’s protocol.
- Picrosirius red stain: slides were stained with Weigert hematoxylin (Sigma) for 8 minutes, rinsed for 10 minutes in running tap water, then incubated for 1 hour in a 0.1% solution of Direct Red 80 (Sigma) in saturated aqueous picric acid (1.3%). Destaining was accomplished by incubating slides in two rinses of 0.5% aqueous acetic acid for 1 minute each. Low-power images were taken using both a standard bright field filter and a polarizing filter. At least 9 images were collected per heart, capturing a large section of the left ventricle. Large vessels were avoided because of their high collagen content. ImageJ was used to threshold and measure area of each image, and percent of total ara occupied by fibrotic tissue was used for analyses.

Ventricular cardiomyocyte isolation: 12-13 weeks old mice were anesthetized by sodium pentobarbital (50 mg/mL) with Heparin (55 units/mL) through IV injection (1 mL/1 kg). Hearts were excised, perfused retroaortically (Langendorff), and enzymatically digested with a mixture of collagenase (Type 2) (250 units/mL), hyaluronidase (0.01%), and protease type XIV (0.0025%) in a modified Tyrode’s solution (0.1mM CaCl2, 10 mM BDM). Dissociated cardiomyocytes were washed three times in Joklik MEM (Sigma-Aldrich) with 1 % Pen/Strep and 1X ITS with increasing Ca2+ (0.25, 0.5, 0.75 mM). Isolated myocytes were cultured with media consisting a 50:50 mix of DMEM and F10 media with 1% Pen/Strep and 1X ITS.

Western Blots: Hearts were homogenized in RIPA buffer (see supplemental experimental procedures). Total protein concentration was determined by BCA (Pierce). 30 µg of protein was reduced and separated by SDS-PAGE on 12% bis-tris polyacrylamide gels in MOPS buffer (Bio-Rad), then transferred to 0.2 µm PVDF (Bio-Rad). Antibodies used are described in supplemental experimental procedures. Blots were developed using ECL Plus Western Blotting Detection System (GE Healthcare) and imaged digitally on a ChemiDoc MP imaging system (Bio-Rad). Blots were stripped no more than two times using Restore Stripping Buffer (Thermo). Densitometry was performed using NIH ImageJ software. Densities of bands detected by phospho-antibodies were normalized to densities of bands detected by phospho-independent antibodies in same lane. Phospho-independent antibody densities were normalized to αTubulin or Na/K-ATPase band in same lane.

Antibodies: Cell signaling (Danvers, MA, USA): P-S6 (ser235/236, 4858), S6 (2217), P-4EBP1 (ser65, 9451), 4EBP1 (9644), P-Akt (ser473, 4060), Akt (4691), P-mTOR (ser2448, 5536),
mTOR (2983), P-AMPKα (thr172, 2535), P-p44/42 MAPK (thr202/tyr204, 4370), p44/42 MAPK (4695), Rheb (13879). Abcam (Cambridge, UK): Na/K-ATPase (ab76020). Santa Cruz (Dallas, TX, USA): AMPKα (sc-25792). Sigma: αTubulin (T5168). Secondary antibodies used were HRP-donkey anti-mouse and HRP-donkey anti-rabbit (NA931 and NA934, respectively; GE Healthcare, Pittsburg, PA, USA).

Quantitative PCR: RNA was isolated using TRIzol (Ambion) according to manufacturer’s protocol. Random-primed first-strand cDNA synthesis was performed using 2 µg RNA (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems) according to manufacturer’s protocol. Quantitative PCR was performed using SYBR green PCR master mix (Invitrogen) on a real-time CFX384 PCR detection system (Bio-Rad). Primers were designed with Primer3 or were ordered as predesigned sets (IDT). Primer sequences are available upon request.

Injections: AAVs were injected through the right external jugular vein to minimize loss of vector in the circulation that could occur with injections at a site more distal to the heart. AAV doses for each mouse were calculated based on body mass, with a dose of 7.5e10 vg/g body mass for miRNA-mediated knockdown experiments and 3e10 vg/g body mass for overexpression experiments. Mice were anesthetized with 2.5% isoflurane and a small incision was made over the right clavicle. The right external jugular vein and right pectoral muscle were identified, and a 27g insulin needle containing the appropriate AAV dose was inserted rostrally through the pectoral muscle into the jugular vein. Injection was confirmed visually.

CT.AAV biodistribution: tissue was fixed in 4% PFA, cryoprotected in 30% sucrose, then embedded in OCT using 2-methylbutane in liquid nitrogen for cooling. Blocks were sectioned to 10 µm by cryostat, allowed to melt to the slides, and mounted with Fluorogel (Electron Microscopy Sciences).

Immunohistochemistry: 5-7 µm sections of PFA-fixed, paraffin-embedded heart tissues were rehydrated and subjected to heat-induced epitope retrieval for 3 minutes using a pressure cooker and sodium citrate buffer (10 mM Sodium citrate, 0.05% Tween 20, pH 6). Sections were blocked in H2O2 following incubation in primary antibody, and developed using DAB.

Membrane isolation: Total cell membranes were isolated using Mem-PER Plus Membrane Protein Extraction kit (Thermo) according to manufacturer’s instructions.

TUNEL Immunostaining: Mice hearts were postfixed overnight in 4% PFA, and 6-μm thick sections were collected. TUNEL staining was performed on 6-μm-thick heart sections using TACS® 2 TdT DAB Kit (Trevigen, Gaithersburg, MD, USA). An investigator blinded to the mouse genotype and treatments assessed the number of TUNEL-positive cells. Images were captured using an Olympus BX60 light microscope and DP70 camera with Olympus DP Controller software (Olympus, Melville, NY, USA).

Food consumption: Mice were housed individually in Comprehensive Laboratory Animal Monitoring System cages (CLAMS; Columbus Instruments, Columbus, OH, USA) for 48 hours. Food was provided ad libitum from a dispenser on a scale and measurements were taken every twenty minutes. The first 24 hours were considered a habituation period and data from this block were discarded.

Heart Homogenization: For RNA extraction, hearts were homogenized in 250 ml TRIZol using a bead mill (Eppendorf). An additional 750 ml TRIZol was then added to homogenates, and samples were incubated for 30 minutes at 4°C with shaking. For protein extractions, hearts were homogenized by bead mill in TBS (50 mM Tris, 150 mM NaCl), with protease inhibitors (Complete; Roche Applied Science) and phosphatase inhibitors (PhosphoSTOP; Roche). Concentrated 1.33X RIPA buffer was then added to bring volume to 1 ml and final
concentrations of 50 mM Tris, 150 mM NaCl, 1% Triton-X100, 0.1% SDS, 0.5% sodium deoxycholate, with protease and phosphatase inhibitors (Roche). Samples were incubated for 1 hour with shaking at 4°C, then clarified by centrifugation at 18,000 x g for 15 minutes.

Virus Titers: CT.AAV.U6.mi2.1 2.23E+13 vg/ml; CT.AAV.U6.miCTRL 2.63E+13 vg/ml; CT.AAV.CAG.caRheb 1.17E+13 vg/ml; CT.AAV.CAG.GFP 1.03E+13 vg/ml.
Figure S1. Additional cardiac phenotypes in HD mouse models. Related to Figures 1 and 2.

A-B. (A) Tibia lengths (mm) and (B) heart mass/body mass ratios (mg/g) in N171-82Q mice across lifespan: 6 week-old (presymptomatic, n ≥ 6), 10 week-old (early-stage disease, n ≥ 10), 14 week-old (mid-stage disease, n ≥ 8), and 18 week-old (late-stage disease, n ≥ 8).

C. Comparison of heart mass changes to body mass changes in N171-82Q mice across lifespan.

D-E. (D) Tibia lengths (mm) and (E) heart mass/body mass ratios (mg/g) in zQ175 mice at 12 months of age (n ≥ 10).

F. TUNEL stain of representative N171-82Q heart sections (n = 4) and positive control. Apoptotic nuclei stain black. Scale bars = 50 µm.

G-H. Quantitative PCR analyses for (F) Anp and (G) Bnp in N171-82Q mice at 6 (n ≥ 6), 10 (n ≥ 7), 14 (n ≥ 8), and 18 (n ≥ 8) weeks of age, and in zQ175 mice at 12 months of age (n ≥ 10). All genes were normalized to expression of TATA-binding protein (Tbp) mRNA as an internal control and are graphed relative to WT levels.

Student’s T-test with Bonferroni correction, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Error bars represent standard error of the mean.
Figure S2. Additional changes observed in stressed HD and WT hearts. Related to Figure 5.

A. Transthoracic echocardiography measurements of left ventricular end diastolic volume (LVEDV), left ventricular end systolic volume (LVESV), cardiac output (CO), and ejection fraction (EF) in ISO- or saline-treated N171-82Q mice and WT littermates at trial days 0, 5, and 12. Graphs represent changes from baseline. n ≥ 5 for each group.

B. Heart hypertrophy measured by heart mass/tibia length in ISO- or saline-treated N171-82Q mice and WT littermates. n ≥ 8 for all groups.

C. Representative images of Sirius red staining for collagen in WT and HD mice treated with ISO or saline.

D. Relative expression of Bax and Bcl2 genes in WT and HD mice treated with ISO or saline. n ≥ 5 for each group.

E. Wet/Dry lung mass ratio in WT and HD mice treated with ISO or saline. n ≥ 5 for each group.

F. Western blot analysis of phosphorylated and total S6, P-4EBP1, and 4EBP1 in WT and HD mice treated with ISO or saline. n ≥ 5 for each group.
C. Representative micrographs of picrosirius red-stained hearts from N171-82Q or WT mice treated with either isoprenaline (ISO) or saline. Image set on left is standard bright field views, image set on right is the same window viewed through a polarized filter. Scale bars = 40 µm.

D. qPCR for markers of apoptosis, Bax and Bcl2, in N171-82Q and WT hearts treated with either ISO or saline. Expression levels were normalized to Tbp and are expressed relative to WT-Saline expression levels for each gene. n ≥ 7 for each group.

E. Wet/dry lung mass ratios from N171-82Q or WT mice treated with either ISO or saline. n ≥ 7 for each group.

F. Representative western blot and quantitation of P-S6/S6 and P-4EBP1/4EBP1 levels in N171-82Q or WT hearts treated with either ISO or saline for 14 days. Data are graphed relative to levels from saline-treated WT samples. n ≥ 6 for all groups.

One-way ANOVA with Sidak’s multiple comparison test (A) or Tukey’s post-hoc analysis (B-F); *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Error bars represent standard error of the mean (SEM).
Figure S3. Additional changes in stressed N171-82Q hearts expressing caRheb or GFP. Related to Figure 6.
A. Western blot confirmation of caRheb and GFP expression in N171-82Q hearts injected with either CT.AAV.GFP (G) or CT.AAV.caRheb (R) used for cardiac stress studies.

B. Transthoracic echocardiography measurements in N171-82Q mice injected with either CT.AAV.GFP or CT.AAV.caRheb prior to infusion with either ISO or saline at days 0, 5, and 12 post-treatment. Data are graphed as changes from baseline. LVEDV: left ventricular end diastolic volume; LVESV: left ventricular end systolic volume; CO: cardiac output; EF: ejection fraction. n ≥ 4 for each group.

C. Heart mass/tibia length as an indicator of heart hypertrophy in N171-82Q mice injected with either CT.AAV.GFP or CT.AAV.caRheb and treated with isoprenaline (ISO) or saline for 14 days. n ≥ 7 for all groups.

D. Representative micrographs of picrosirius red-stained hearts from N171-82Q mice injected with either CT.AAV.caRheb or CT.AAV.GFP, then treated with either isoprenaline (ISO) or saline for 14 days. Image set on left is standard bright field views, image set on right is the same window viewed through a polarized filter. Scale bars = 40 µm.

E. qPCR for markers of apoptosis, Bax and Bcl2, in N171-82Q hearts expressing either caRheb or GFP and treated with either ISO or saline. Expression levels were normalized to Tbp and are expressed relative to WT-Saline expression levels for each gene. n ≥ 5 for each group.

F. Wet/dry lung mass ratios from N171-82Q injected with CT.AAV.caRheb or CT.AAV.GFP and treated with either ISO or saline for 14 days. n ≥ 5 for each group.

G. Representative western blot and quantitation of P-S6 and P-4EBP1 levels in N171-82Q hearts transduced with either GFP or caRheb and treated with either ISO or saline for 14 days. Data are graphed relative to GFP-Saline samples. n ≥ 6 for all groups.

One-way ANOVA with Sidak’s multiple comparison test (B) or Tukey’s post-hoc analysis (C-G); *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
Figure S4. Additional upstream regulators of mTORC1 in HD mouse hearts. Related to Figure 7.

A. Representative western blot and graphs representing densitometry quantitation for P-Erk and total Erk in N171-82Q mouse hearts at 6 weeks (n ≥ 6), 10 weeks (n ≥ 10), 14 weeks (n ≥ 8), and 18 weeks (n ≥ 8) of age and zQ175 mouse hearts at 12 months (n ≥ 6) of age. Graphs represent P-Erk1 and P-Erk2 densities normalized to total Erk1 and total Erk2, respectively, relative to WT.

B. Serum insulin levels in N171-82Q mice at 6 (n ≥ 7) and 14 (n ≥ 5) weeks of age and in zQ175 mice at 12 months of age (n ≥ 7) relative to WT.

C. Food consumption by N171-82Q mice at 9 (n ≥ 6), 13 (n ≥ 3), and 17 (n ≥ 6) weeks of age. Graphs represent food consumed in grams per 25 g body mass and are stratified into dark cycle, light cycle, or total (combined light and dark).

Student’s T-test, *p ≤ 0.05. Error bars represent standard error of the mean.
Figure S5. Expression of FoxO family transcription factors and target genes in HD murine model hearts. Related to Figure 7.

A-C. Analyses of mRNA expression in N171-82Q hearts at 6 (n ≥ 6), 10 (n ≥ 7), 14 (n ≥ 8), and 18 (n ≥ 8) weeks of age, and in zQ175 mice at 12 months of age (n ≥ 10) by qPCR. Expression was queried for the following genes: (A) FoxO1; (B) FoxO3; (C) Bnip3. All genes were normalized to Tbp as an internal control and expressed relative to WT.

Student’s T-test, *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001. Error bars represent standard error of the mean (SEM).
Figure S6. Additional analyses of caRheb localization in N171-82Q hearts. Related to Figure 7.

A. Representative western blot and quantitation of Rheb band densities in 6 week-old N171-82Q hearts following membrane extraction. Na/K-ATPase was used as loading control for membrane fractions, αTubulin was used as loading control for cytosolic fractions. n ≥ 5 for all groups. Student’s T-test, *p ≤ 0.05. Error bars represent standard error of the mean (SEM).