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Temsirolimus activates autophagy and ameliorates cardiomyopathy caused by lamin A/C gene mutation

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Abstract

Mutations in the lamin A/C gene (*LMNA*) encoding A-type lamins cause a diverse range of diseases collectively called laminopathies, the most common of which is dilated cardiomyopathy. Emerging evidence suggests that *LMNA* mutations cause disease by altering cell signaling pathways but the specific mechanisms involved are poorly understood. Here we show that AKT-mTOR pathway is hyperactivated in hearts of mice with cardiomyopathy caused by *Lmna* mutation and that *in vivo* administration of the rapamycin analog temsirolimus prevents deterioration of cardiac function. We also show defective autophagy in hearts of these mice and that improvement in heart function induced by pharmacological interventions is correlated with enhanced autophagy. These findings provide a rationale for a novel treatment of *LMNA* cardiomyopathy and implicate defective autophagy as a pathogenic mechanism of cardiomyopathy arising from *LMNA* mutation.

Introduction

Laminopathies are a diverse group of diseases arising from mutations in *LMNA*, the gene that encodes the A-type lamins A and C (1). A-type lamins are intermediate filament proteins of the nuclear lamina, a proteinaceous meshwork lining the inner nuclear membrane. Although A-type lamins are expressed in most differentiated mammalian

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Author contributions: J.C.C. and H.J.W. generated the hypotheses and designed experiments. J.C.C. performed experiments, generated data in all figures and Supplementary Data. A.M. and W.W. designed and performed experiments treating and analyzing *Lmna*^{H222P/H222P} mice in Fig. 2. S.I. and S.H. performed echocardiographic analysis in Fig. 2. J.P.M. designed experiments using isolated cardiomyocytes in Fig. 4. H.J.W. checked and reviewed data analyses. J.C.C. and H.J.W. wrote the manuscript. All authors reviewed and edited the manuscript prior to submission.

Competing interests: H.J.W. and A.M. are inventors on a pending patent application (PCT/US09/42614) on methods for treating and/or preventing cardiomyopathies by ERK and JNK inhibition filed by the Trustees of Columbia University in the City of New York. H.J.W. and J.C.C. are inventors on a provisional patent application on the use of rapamycin and rapamycin analogs for the treatment of dilated cardiomyopathies currently planned for filing by the Trustees of Columbia University in the City of New York.

Supplementary Material

Fig. S1. Effect of temsirolimus on AKT phosphorylation.

Fig. S2. p62 mRNA expression in hearts of *Lmna*^{H222P/H222P} mice.

Fig. S3. Analysis of FoxO3a signaling in hearts of *Lmna*^{H222P/H222P} mice.

Fig. S4. Effect of temsirolimus on lamin A/C, beclin1 and phosphorylated ERK1/2 expression.

Table S1. Heart tissue samples from human subjects.

Table S2. Primers used for qPCR analyses.

Table S3. Echocardiography data with additional statistics.

somatic cells, specific mutations in *LMNA* lead to tissue-selective diseases affecting striated muscle, adipose tissue, or peripheral nerve, or more generalized diseases with features of accelerated aging, such as Hutchinson-Gilford progeria syndrome (HGPS). The most prevalent laminopathy is dilated cardiomyopathy (herein referred to as *LMNA* cardiomyopathy) with variable skeletal muscle involvement, which includes autosomal Emery-Dreifuss muscular dystrophy and limb girdle muscular dystrophy 1B. Patients with *LMNA* cardiomyopathy have higher rates of deadly arrhythmias and a relatively early onset of heart failure compared to individuals with most other inherited dilated cardiomyopathies (2). The pleiotropic nature of laminopathies, as well the mechanisms involved in the pathogenesis of specific diseases such as *LMNA* cardiomyopathy, are poorly understood.

Emerging evidence suggests that *LMNA* mutations that cause abnormalities in the nuclear lamina lead to dysregulation of signaling pathways that underlie disease pathogenesis (1). For example, impaired canonical Wnt signaling contributes to HGPS by reducing the expression of genes encoding extracellular matrix components (3). Notably, inducing autophagic clearance of the lamin A variant responsible for HGPS with the autophagy activating compound rapamycin improves cellular phenotypes associated with the disease (4). In *LMNA* cardiomyopathy, ERK1/2 signaling is hyperactivated in the hearts of human subjects with *LMNA* cardiomyopathy and in *Lmna*^{H222P/H222P} mice, an animal model of the disease (5,6). Reducing ERK1/2 signaling to basal levels in hearts of *Lmna*^{H222P/H222P} mice ameliorates cardiomyopathy (6-8), demonstrating that ERK1/2 hyperactivation contributes to pathogenesis.

AKT signaling, which is predominantly triggered by insulin or insulin-like growth factor, is also implicated in the development of cardiomyopathy. Although AKT is generally accepted to promote cell survival in response to acute ischemic stress (9,10), more recent evidence suggests that AKT activation also contributes to the pathogenesis of cardiomyopathy and that blocking downstream signaling prevents the disease (11,12). Concurrent activation of both ERK1/2 and AKT signaling has also been documented in failing hearts of human subjects (13,14) and in hearts from mice with cardiomyopathy induced by overexpression of Gα subunit (15). We therefore have investigated the involvement of AKT signaling in the pathogenesis of *LMNA* cardiomyopathy.

Results

AKT/mTOR signaling is enhanced in *LMNA* cardiomyopathy

We examined AKT phosphorylation, which is indicative of its activation, in hearts of wild-type and *Lmna*^{H222P/H222P} mice that develop cardiomyopathy (16) at 4, 8, 12 and 16 weeks of age (Fig. 1A). To ensure that observed phenotypes are not secondary to the development of cardiomyopathy, we chose 4 weeks as our earliest timepoint since male *Lmna*^{H222P/H222P} mice do not develop detectable cardiac abnormalities until 8 weeks of age (16). Full activation of AKT requires phosphorylation on two separate sites, threonine 308 (T308) and serine 473 (S473). As early as 4 weeks of age, we detected increased AKT phosphorylation on both S473 and T308 (Fig. 1A). As the mice aged, AKT phosphorylation (S473 and T308) in hearts increased further peaking at 12 weeks of age (Fig. 1A).

Activated AKT activates mTORC1 (17-22), which contains mTOR, a protein kinase forming two functionally distinct complexes, mTORC1 and mTORC2. mTORC1 is a central regulator of metabolism and cell growth (23) implicated in the pathogenesis cardiomyopathy (11,12,24-26) and mTORC2 catalyzes phosphorylation of AKT on S473 (23). To determine whether mTOR activation was concurrent with AKT activation, we assessed its phosphorylation in the same heart samples. Similar to AKT phosphorylation, we observed enhanced mTOR phosphorylation at 4 weeks that increased further with age (Fig. 1A). We

observed increased levels of phosphorylated ribosomal protein S6, a downstream target of mTORC1 starting at 8 weeks, confirming mTORC1 activation. These results demonstrate activation of AKT/mTOR signaling in hearts of *Lmna*^{H222P/H222P} mice at stages prior to the onset of clinically detectable cardiomyopathy.

Activated ERK1/2 can catalyze an inhibitory phosphorylation on tuberous sclerosis complex (27), a repressor of mTORC1, hence potentially activating it. Because ERK1/2 is hyperactivated in hearts of *Lmna*^{H222P/H222P} mice (5), we sought to determine whether enhanced ERK1/2 signaling contributed to the increased phosphorylation of mTOR. We examined mTOR phosphorylation in hearts of *Lmna*^{H222P/H222P} mice treated systemically with selumetinib (1 mg kg⁻¹ d⁻¹), an inhibitor of ERK1/2-activating kinase MEK1/2, from 16 weeks of age to 20 weeks. This dose of selumetinib blocks cardiac ERK1/2 phosphorylation and improves heart function in *Lmna*^{H222P/H222P} mice (6). Selumetinib reduced mTOR phosphorylation in heart relative to controls given dimethylsulfoxide (DMSO) (Fig. 1B). We also observed consistent reduction in AKT phosphorylation after ERK1/2 inhibition (Fig. 1B), which may further contribute to the reduced mTOR phosphorylation. These results show that ERK1/2 hyperactivation contributes to the enhancement of mTOR phosphorylation in hearts of *Lmna*^{H222P/H222P} mice.

To test whether the changes we observed in hearts of *Lmna*^{H222P/H222P} mice also occur in human disease, we examined phosphorylated AKT levels in left ventricular tissue from human subjects with confirmed mutations in the *LMNA* gene (Supplementary Table 1). Compared to controls, we observed enhanced AKT phosphorylation (on T308 and S473) in ventricular tissue from human subjects with *LMNA* cardiomyopathy (Fig. 1C). This observation established that enhanced AKT-mTORC1 signaling is a feature of *LMNA* cardiomyopathy in humans.

Given the enhanced AKT-mTORC1 signaling in hearts of *Lmna*^{H222P/H222P} mice, we hypothesized that reducing mTORC1 activity would prevent progression of cardiomyopathy. We blocked mTORC1 in 14 week-old *Lmna*^{H222P/H222P} mice with daily intraperitoneal injections of temsirolimus (5 mg kg⁻¹). After two weeks of treatment, mice were analyzed by echocardiography and sacrificed for biochemical analyses. Temsirolimus reduced phosphorylated mTOR and S6 in hearts compared to DMSO placebo (Fig. 2A). Previously reported effects of temsirolimus on AKT phosphorylation (increased and decreased phosphorylation on T308 and S473, respectively) (28-30) were also observed, further demonstrating a pharmacological effect (Fig. S1A and S1B). At 16 weeks, *Lmna*^{H222P/H222P} mice exhibit significantly increased left ventricular end-diastolic and end-systolic diameters and markedly decreased fractional shortening compared to wild-type mice (7,8,16). After temsirolimus treatment, overall heart size was reduced compared to placebo controls (Fig. 2B). M-mode echocardiography showed that left ventricular diameters were significantly smaller and fractional shortening significantly greater in temsirolimus-treated mice than controls (Fig. 2C, D). Temsirolimus also reduced mRNA levels of *NppA* and *NppB*, which encode natriuretic peptide precursors that stimulate vasodilation and vascular fluid egress to compensate for ventricular dilatation, but not *Colla1*, *Colla2* or *Fnl* mRNAs encoding collagens and fibronectin involved in fibrosis (Fig. 2E). Levels of atrial natriuretic peptide, encoded by *NppA*, were also decreased (Fig. 2F). Hence, mTORC1 inhibition improves left ventricular diameters and apparent contractility in *Lmna*^{H222P/H222P} mice but may not prevent cardiac fibrosis.

Autophagy is impaired in *LMNA* cardiomyopathy

Given that mTORC1 blockade in hearts of *Lmna*^{H222P/H222P} mice prevented progression of cardiomyopathy, we explored putative pathogenic mechanisms engendered by activated mTORC1. We focused on macroautophagy (autophagy), which involves lysosome-

dependent degradation of cytoplasmic cargo enclosed in a double membrane vesicle termed the autophagosome (31,32). As mTORC1 inhibits autophagy and defective autophagy has been linked to cardiomyopathy (33-35), we explored autophagic responses in hearts of *Lmna*^{H222P/H222P} mice.

Autophagic flux can be broadly categorized into two general steps: 1) vesicle nucleation and formation of autophagosomes and 2) lysosome-mediated autophagosome degradation. We assessed the conversion of microtubule-associated protein-1 light chain 3 β from its non-lipidated (LC3B-I) to lipidated form (LC3B-II) during autophagosome formation (31,36). Hearts from 12 and 16 week-old *Lmna*^{H222P/H222P} mice exhibited slightly variable but generally reduced steady-state levels of LC3B-II compared to wild-type mice (Fig. 3A and 3B). As autophagic flux is dynamic, reduced LC3B-II levels can indicate either impaired formation or enhanced degradation of autophagosomes. Therefore, we assessed p62/SQSTM1 (p62) expression, an LC3B-binding protein that is degraded along with LC3B-II during vesicle degradation (37). Enhanced p62 expression was observed at 12 weeks that increased further by 16 weeks (Fig. 3A and 3B). Increased p62 levels did not correlate with its mRNA expression (Fig. S2). Accumulation of p62 coincident with reduced LC3B-II is suggestive of defective autophagosome formation rather than rapid degradation. This occurred despite increased expression of beclin1, an activator of autophagy necessary for vesicle nucleation, at 16 weeks (Fig. 3A) (32). These data suggest that despite increased beclin1 expression autophagosome formation was progressively impaired in hearts of *Lmna*^{H222P/H222P} mice.

To further verify that autophagosome formation is impaired *in vivo*, we treated 15 week-old *Lmna*^{H222P/H222P} mice with chloroquine (50 mg kg⁻¹ d⁻¹), a lysosomal inhibitor that reduces autophagosome degradation (38). Administration of chloroquine for 10 days resulted in a significant increase in LC3B-II and p62 in hearts of wild-type mice (Fig. 3C), demonstrating autophagosome accumulation. However, chloroquine had insignificant effects on LC3B-II levels in hearts of *Lmna*^{H222P/H222P} mice (Fig. 3C), confirming that autophagosome formation is impaired. Likewise, no significant change was observed in p62 levels in hearts of *Lmna*^{H222P/H222P} mice, which was elevated prior to chloroquine administration. To further characterize the impairment, we examined autophagic responses in hearts of wild-type and *Lmna*^{H222P/H222P} mice fasted for 24 hr. In hearts of wild-type mice, fasting led to a significant increase in LC3B-II whereas p62 levels, although decreased relative to fed wild-type mice, were not significantly different (Fig. 3D). No significant differences were observed in LC3B-II and p62 levels between fed and fasted *Lmna*^{H222P/H222P} mice, suggesting that fasting-induced autophagic responses were also defective in hearts of *Lmna*^{H222P/H222P} mice.

To eliminate the influence of cardiac fibroblasts, we assessed autophagic responses in isolated ventricular cardiomyocytes from 12 week-old *Lmna*^{H222P/H222P} mice. No phenotypic differences were observed between cardiomyocytes from wild-type and *Lmna*^{H222P/H222P} mice when assessed by light microscopy (Fig. 4A). We then deprived cardiomyocytes of glucose for 2 hr and 4 hr and examined LC3B-II and p62 levels. Glucose-deprived cardiomyocytes from wild-type mice exhibited enhanced LC3B-II expression with minor decreases in p62, whereas both basal and glucose-deprived LC3B-II levels in cardiomyocytes from *Lmna*^{H222P/H222P} mice were comparatively reduced (Fig. 4B). Levels of p62 were also enhanced in *Lmna*^{H222P/H222P} cardiomyocytes, indicative of impaired autophagic flux, and were unaffected by glucose deprivation. These results establish defective autophagy as features of cardiomyocytes of *Lmna*^{H222P/H222P} mice. To test whether similar phenotypes exist in the human disease, we examined LC3B-II and p62 levels in left ventricular tissue from human subjects. Virtually no LC3B-II was detected in tissue from human subjects with *LMNA* cardiomyopathy compared to unaffected controls

(Fig. 4C). Moreover, we observed increased p62 levels in hearts with *LMNA* cardiomyopathy. These results show that autophagy is impaired in hearts with *LMNA* cardiomyopathy and support the hypothesis that defective autophagy underlies *LMNA* cardiomyopathy pathogenesis.

AKT activation can inhibit autophagy via inhibitory phosphorylation of FoxO3a (39,40). We did not observe significant differences in phosphorylated FoxO3a or expression of mRNAs encoded by genes associated with a FoxO3a-activated autophagy in hearts of wild-type and *Lmna*^{H222P/H222P} mice (Fig. S3A and S3B). This ruled out the possibility that AKT inhibited autophagy via FoxO3a.

Temsirolimus reactivates autophagy

To test whether the beneficial effect of temsirolimus in the heart may be mediated by enhancing autophagy, we measured LC3B-II and p62 expression in hearts of temsirolimus-treated *Lmna*^{H222P/H222P} mice. Compared to placebo controls, hearts from temsirolimus-treated mice exhibited increased LC3B-II and reduced p62 expression (Fig. 4D), an expression profile indicative of enhanced autophagy. Temsirolimus did not alter the expression of beclin1 or phosphorylated ERK1/2 (Fig. S4).

Defective autophagy should lead to accumulation of ubiquitinated proteins (32), which should be degraded by temsirolimus-induced autophagy. To verify if this is the case in the hearts of *Lmna*^{H222P/H222P} mice, we performed immunofluorescence microscopy on heart sections from 16 week-old wild-type, DMSO-treated *Lmna*^{H222P/H222P}, and temsirolimus-treated *Lmna*^{H222P/H222P} mice with anti-ubiquitin antibodies. Antibodies against α -actin, which detect the I-band of sarcomeres, were used to identify cardiomyocytes. In hearts from wild-type mice, we observed mostly diffuse fluorescence that co-localized with α -actin labeling (Fig. 4E). In contrast, we observed large aggregates of ubiquitinated proteins in hearts of DMSO-treated *Lmna*^{H222P/H222P} mice and temsirolimus treatment noticeably reduced these aggregates (Fig. 4E). Similarly, insoluble aggregates of lamin A/C, in the form of intranuclear foci, have been proposed to contribute to disease pathogenesis of laminopathies (4). We therefore labeled cardiac tissue with anti-lamin A/C antibodies to assess whether lamin A/C aggregates were visible in the hearts of *Lmna*^{H222P/H222P} mice. We did not observe obvious lamin A/C aggregates and temsirolimus had no observable effects on lamin A/C expression (Fig. 4F). This result was confirmed by Western blot analysis demonstrating comparable lamin A/C expression in hearts of DMSO and temsirolimus-treated *Lmna*^{H222P/H222P} mice (Fig S4). However, consistent with previous studies (7, 8), we observed irregular, misshapen nuclei in hearts of *Lmna*^{H222P/H222P} mice and temsirolimus did not reverse or improve this irregular nuclear morphology (Fig. 4F).

Reducing ERK1/2 signaling also ameliorates cardiomyopathy in *Lmna*^{H222P/H222P} mice (6-8). To verify whether this was associated with enhanced autophagy, analogous to that observed with temsirolimus treatment, we measured LC3B-II and p62 expression in hearts of selumetinib-treated *Lmna*^{H222P/H222P} mice. Selumetinib treatment increased the level of LC3B-II while reducing p62 in hearts of *Lmna*^{H222P/H222P} mice (Fig. 4G), consistent with enhanced autophagy. Taken together, our results link reactivation of autophagy to improved cardiac function following ERK1/2 or mTORC1 blockade. They further suggest that temsirolimus may be beneficial as a treatment for *LMNA* cardiomyopathy.

Discussion

Our findings support a model for the pathogenesis of *LMNA* cardiomyopathy (Fig. 4H) in which the *Lmna*^{H222P} mutation results in aberrant activation of both the ERK1/2 and AKT signaling cascades that converge on mTORC1. Activated mTORC1 inhibits autophagic

responses and reduces tolerance to a putative energy deficit. In this scenario, the heart is unable to compensate for increased or fluctuating energy demand and over time develops muscle damage and dilated cardiomyopathy. The beneficial effects of MEK1/2 inhibitors such as selumetinib and PD98059 in *LMNA* cardiomyopathy (6-8) may be mediated in part by reducing mTORC1 activities. Moreover, through undefined mechanisms, MEK1/2 inhibition also reduces AKT phosphorylation, which may contribute to further decrease mTORC1 activities. Temsirolimus and other rapamycin analogs similarly block mTORC1 activity and could reactivate autophagy and prevent the progression of cardiomyopathy.

The exact mechanisms that cause dysregulated AKT/mTORC1 and MEK/ERK1/2 signaling arising from defects in nuclear lamins remain to be elucidated. A-type lamins control ERK1/2 signaling output by acting as a molecular scaffold that allows efficient interaction between ERK1/2 and c-Fos (41). Perhaps ERK1/2 feedback inhibitory mechanisms are disrupted by amino acid substitutions in A-type lamins that cause cardiomyopathy. Alternatively, extracellular factors may play a role. For example, Gene Ontology analysis of mRNA expression in hearts from *Lmna*^{H222P/H222P} mice hinted at the involvement the insulin-like growth factor signaling pathway (5). Intracellular propagation of insulin-like growth factor signaling is predominantly mediated by triggering of both the AKT and ERK1/2 cascades (42).

As pathways that mediate cell growth and proliferation, both AKT and ERK1/2 signaling have been implicated in hypertrophic cardiomyopathy, which exhibits abnormal thickening of the myocardium. The demonstration that AKT and ERK1/2 are hyperactivated in *LMNA* cardiomyopathy, which is characterized by left ventricular dilatation with a thinned left ventricular wall, suggest that hypertrophic and dilated forms of cardiomyopathy share a putative pathogenic mechanism(s) emanating from deregulated cell signaling. Moreover, the convergence of the AKT and ERK1/2 cascades on mTORC1 imply a central role for mTORC1 in cardiomyopathy. Indeed, the involvement of mTOR in cardiac pathophysiology is evolutionarily conserved from fruit flies to mammals (24,43). Enhanced mTOR signaling is observed in mouse models of hypertrophic cardiomyopathy and inhibition of mTORC1 with rapamycin ameliorates the disease (11,24-26). However, cardiac-specific ablation of mTOR (44) or Raptor (45), an essential component of mTORC1, results in dilated cardiomyopathy, suggesting that a threshold level of mTORC1 activity is necessary for proper cardiac homeostasis and response to physiological stress.

We have also shown that pharmacological mTORC1 inhibition reactivates autophagy and prevents cardiac damage in a mouse model of *LMNA* dilated cardiomyopathy. Furthermore, we have shown that inhibiting ERK1/2 signaling, previously shown to be a beneficial in the same model of cardiomyopathy, enhances autophagy in the heart. On the basis of these findings, it could be hypothesized that combining MEK1/2 and mTORC1 inhibitors to treat *LMNA* cardiomyopathy may be synergistic, given that ERK1/2 and mTORC1 trigger divergent mechanisms that contribute to *LMNA* cardiomyopathy (for example, induction of genes involved in fibrosis elicited by ERK1/2 hyperactivation). In this regard, we performed a pilot study with a small group of mice (n=3) that were treated with temsirolimus alone or in combination with selumetinib for two weeks from 18 to 20 weeks of age. This preliminary study showed no apparent benefit of adding selumetinib to temsirolimus (fractional shortening of 19.1 +/- 6.7 SD for temsirolimus alone compared to 14.5 +/- 4.6 SD for combination treatment with $p=0.38$). Future studies optimizing drug doses, treatment duration, and the age of initial treatment will be required to definitively assess therapeutic benefit of a combination treatment or to improve treatment outcomes by reduced dosing of drugs to avoid adverse events associated with higher doses. Furthermore, while temsirolimus alone clearly improves heart function in *Lmna*^{H222P/H222P} mice, more comprehensive

experiments will be required to determine if it prolongs survival in these mice, which also develop significant skeletal muscle disease at later ages (16).

MATERIALS AND METHODS

Animals

The Columbia University Medical Center Institutional Animal Care and Use Committee approved all protocols using vertebrate animals and the investigators adhered to the NIH Guide for the Care and Use of Laboratory Animals. *Lmna*^{H222P/H222P} mice were generated and genotyped as described (16). Only male mice were used. Selumetinib treatment has been described elsewhere (6). For temsirolimus treatment, 14 week-old *Lmna*^{H222P/H222P} mice were given 5 mg kg⁻¹ d⁻¹ temsirolimus or DMSO for two weeks by intraperitoneal administration. Chloroquine, dissolved in phosphate-buffered saline, was similarly injected into 15 week-old mice (fed *ad libitum*) at 50 mg kg⁻¹ d⁻¹ for 10 days. Autophagy was induced *in vivo* by fasting mice (given only water) for 24 hr.

Human subjects

Heart tissue from human subjects with *LMNA* cardiomyopathy was obtained from Myobank-AFM of the Institut de Myologie. Control human heart samples without patient identifiers were obtained from the National Disease Research Interchange (Table S1). All tissue samples were obtained with appropriate approvals and consent from Institut de Myologie and National Disease Research Interchange (not specifically for this study) and provided without patient identifiers.

Isolation of mouse cardiomyocytes

Cardiomyocytes were isolated using methods as described (46). Briefly, the heart was removed and the aorta cannulated. After Ca²⁺-free buffer was perfused for two minutes, 0.3 mg ml⁻¹ collagenase I/II (Liberase TH, Roche) solution was perfused through the coronary arteries for 6 min with [Ca²⁺] = 12.5 μM. Left ventricular tissue was teased apart and pipetted to release individual cells. After enzymatic dispersion, Ca²⁺ concentration in the buffer containing 3.5 mg ml⁻¹ bovine serum albumin was elevated in three steps up to 400 μM. This method yielded ~99% pure population of cardiomyocytes that were ~80% viable in culture.

RNA isolation and qPCR

Total RNA was isolated using TRIZOL (Invitrogen). Complementary DNAs were generated from 1 μg of RNA primed with 1:1 ratio of random hexameric primers: oligo dT, using Superscript RT II (Invitrogen). qPCR was performed on ABI 7300 Real-Time PCR system (Applied Biosystems) using SYBR green (USB). qPCR primers for *NppA*, *NppB*, *Col1a1*, *Col1a2*, and *Fn1* have been described previously (8). All other primers are listed in Table S2. *Gapdh* and *Tnni3* mRNA were assessed to ensure equal fidelity in enzymatic reactions and equal loading between samples. *Gapdh* mRNA was used as internal control to normalize qPCR unless otherwise indicated. Fold-changes in gene expression were determined by the ΔΔCt method and are presented as fold-change over untreated or wild-type controls.

Protein extraction and Western blot analysis

Whole cell extracts were isolated using RIPA buffer (Sigma) with protease inhibitor cocktail (Roche), 1mM phenylmethanesulfonylfluoride, and 1 mM sodium vanadate. 15 to 30 μg were loaded for SDS-PAGE. The following antibodies were purchased from Cell Signaling Technology: beclin1 (#3738), phospho-ERK1/2 (#9101), LC3B (#2775), phospho-mTOR (Ser2448 #2971), mTOR (#2972), phospho-AKT (Ser473 #4060, Thr308 #4056), phospho-

S6 (Ser240/244 #2215), S6 (#2217), phospho-FoxO3a (Thr32 #9464), FoxO3a (#2497), and ubiquitin (#3936). The following antibodies were purchased from Santa Cruz Biotechnology: ERK1/2 (sc-94), lamin A/C (sc-20681) and α -tubulin (sc-12462-R). Antibodies against p62/SQSTM1 (#610832), α -actin (MA1-21597), AKT (#05-796), and GAPDH (AM4300) were purchased from BD Biosciences, Thermo, Millipore, and Ambion, respectively. Quantification of blots was performed with ImageJ (47), normalized to loading controls as indicated, and presented as arbitrary units or fold change over untreated or wild-type controls.

Transthoracic echocardiography

Lmna^{H222P/H222P} mice were anesthetized with 1-2% isoflurane and placed on a heating pad (37°C) attached to an electrocardiographic monitor. Echocardiography was performed using Vevo 770 imaging system (VisualSonics, Toronto, Canada) equipped with a 30-MHz transducer. Parameters were measured at least three cardiac cycles. A “blinded” echocardiographer, unaware of the treatment received, performed the examinations and interpreted the results.

Statistical analysis

Graphpad (Prism Software Inc) was used to perform statistical analyses. Statistical significance between groups of animals analyzed by echocardiography was analyzed by using corrected parametric test (Welch's *t*-test) with a value of $P < 0.05$ being considered significant. To validate results, a nonparametric test (Wilcoxon-Mann-Whitney test) was performed and concordance checked (Table S3). For all other experiments, a 2-tailed Student's *t*-test was used with a value of $P < 0.05$ considered significant. Values with error bars shown in figures are means $\pm$ standard errors of means. Sample sizes are indicated in the figure legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. AKT/mTORC1 is activated in *LMNA* cardiomyopathy

(A) Western blot analysis of phospho-serine 473 AKT [(pAKT(S473)], phospho-threonine 308 AKT [pAKT(T308)], total AKT, phosphorylated mTOR (pmTOR), total mTOR, phosphorylated S6 (pS6), and total S6 in hearts of 4 to 16 week-old *Lmna*^{H222P/H222P} (*Lmna*^{H222P}) and wild-type (*Lmna*^{WT}) mice (top). Numbers on top of blots denote individual heart samples. Representative blots from two independent experiments. Quantification of pmTOR, pAKT, and pS6 normalized to total mTOR, AKT, and S6, respectively, presented as fold-change over *Lmna*^{WT} (bottom); n = 4.

(B) Western blot of pmTOR, mTOR, pAKT(S473), pAKT(T308), and total AKT, in hearts of 20 week-old *Lmna*^{H222P} mice treated with DMSO or selumetinib for 4 weeks (top). Numbers on top of blots denote individual heart samples. Quantification of pmTOR and pAKT normalized to total mTOR and AKT, respectively, presented as fold-change over DMSO (bottom); n = 6.

(C) Western blot of pAKT (S473 and T308), total AKT and α-tubulin in hearts from human subjects with (*LMNA*^{mt}) or without (*LMNA*^{wt}) *LMNA* cardiomyopathy (top). Numbers on top of blots denote individual heart samples. Quantification of S473 and T308 pAKT normalized to total AKT, presented as fold-change over *LMNA*^{wt} (bottom); n = 3.

Fig. 2. mTORC1 inhibition ameliorates cardiomyopathy in *Lmna*^{H222P/H222P} mice

(A) Western blot of pmTOR, total mTOR, pS6, and total pS6 in hearts of 16 week-old *Lmna*^{H222P/H222P} mice treated with DMSO or temsirolimus (Tamsir) for two weeks (left). Numbers on top of blots denote individual heart samples. Quantification of pmTOR and pS6 normalized to total mTOR and S6, respectively, presented as fold-change over DMSO (right); n = 5.

(B) Representative hearts of 16 week-old *Lmna*^{H222P/H222P} mice treated DMSO or temsirolimus. Scale bar = 1 cm.

(C) Representative M-mode echocardiographic tracings of 16 week-old *Lmna*^{H222P/H222P} mice treated with DMSO or temsirolimus.

(D) Graphic representations and table with numerical values for left ventricular end diastolic diameter (LVEDD), left ventricular end systolic diameter (LVESD) and fractional shortening (FS) of hearts of 16 week-old *Lmna*^{H222P/H222P} mice treated with DMSO or temsirolimus; n = 8.

(E) qPCR analysis on *NppA*, *NppB*, *Col1a1*, *Col1a2* and *Fn1* mRNAs in hearts of 16 week-old *Lmna*^{H222P/H222P} mice treated with DMSO or temsirolimus. *Tnni3* mRNA used as internal control. n = 5.

(F) Western blot of atrial natriuretic peptide (ANP) and GAPDH in hearts of 16 week-old *Lmna*^{H222P/H222P} mice treated with DMSO or temsirolimus (top). Numbers on top of blots denote individual heart samples. Quantification of ANP normalized to GAPDH presented as fold-change over DMSO (bottom); n = 5.

Fig. 3. Autophagy is impaired in hearts of *Lmna*^{H222P/H222P} mice

(A) Western blot of non-lipidated LC3B (LC3B-I), lipidated LC3B (LC3B-II), p62, beclin1, and GAPDH in hearts of 4 to 16 week-old *Lmna*^{H222P/H222P} (*Lmna*^{H222P}) and wild-type (*Lmna*^{WT}) mice (left). Numbers on top of blots denote individual mouse heart samples.

(B) Quantification of LC3B-II and p62 normalized to GAPDH presented as fold-change over *Lmna*^{WT} (right); n = 6; **p* = 0.03; ***p* = 0.002; #*p* = 0.04.

(C) Western blot of LC3B-I, LC3B-II, p62 and α-tubulin in hearts of 16 week-old *Lmna*^{H222P} and *Lmna*^{WT} mice treated with chloroquine (Chloroq) or vehicle (Ctrl) for 10 days (top). Numbers on top of blots denote individual heart samples. Quantification of LC3B-II and p62 normalized to α-tubulin presented as fold-change over Ctrl (bottom); n = 3.

(D) Western blot of LC3B-I, LC3B-II, p62 and α-tubulin in hearts of 16 week-old *Lmna*^{H222P} and *Lmna*^{WT} mice fasted for 24 hr (Fasted) or fed *ad libitum* (Fed) (top). Numbers on top of blots denote individual heart samples. Quantification of LC3B-II and p62 normalized to α-tubulin presented as fold-change over Fed (bottom); n = 3.

Fig. 4. Reactivation of autophagy is associated with improved cardiac function

(A) Photomicrographs of cardiomyocytes from 12 week-old *Lmna*^{H222P/H222P} (*Lmna*^{H222P}) and wild-type (*Lmna*^{WT}) mice; scale bar = 120 μ M.

(B) Representative Western blot of LC3B-I, LC3B-II, p62 and α -tubulin in cardiomyocytes from *Lmna*^{H222P} and *Lmna*^{WT} mice deprived of glucose (Gluc -) for 0, 2 and 4 hr (top). Quantification of LC3B-II and p62 normalized to α -tubulin presented as fold-change over *Lmna*^{WT} (bottom); n = 3; **p* = 0.011; #*p* = 0.046.

(C) Western blot of LC3B-I, LC3B-II, p62 and GAPDH in hearts from human subjects with (*LMNA*^{mt}) or without (*LMNA*^{wt}) *LMNA* cardiomyopathy (top). Numbers on top of blots denote individual samples. Quantification of LC3B-II and p62 normalized to α -tubulin presented as fold-change over *LMNA*^{wt} (bottom); n = 3.

(D) Western blot of LC3B-I, LC3B-II, p62, and α -tubulin in hearts of 16 week-old *Lmna*^{H222P/H222P} mice treated with DMSO or temsirolimus (Temsir) (left). Numbers on top of blots denote individual heart samples. Quantification of LC3B-II and p62 normalized to α -tubulin presented as fold-change over DMSO (right); n = 5.

(E) Immunofluorescence micrographs of heart sections from 16 week-old *Lmna*^{WT}, DMSO-treated *Lmna*^{H222P} and temsirolimus-treated *Lmna*^{H222P} mice probed with antibodies against α -actin and ubiquitin. 4',6-diamidino-2-phenylindole (DAPI) was used as a nuclear counterstain. Representative figures from two independent hearts per group are shown. Scale bar = 30 μ M.

(F) Immunofluorescence micrographs of heart sections from 16 week-old *Lmna*^{WT}, DMSO-treated *Lmna*^{H222P} and temsirolimus-treated *Lmna*^{H222P} mice probed with antibodies against α -actin and lamin A/C. DAPI was used as a nuclear counterstain. Representative figures from two independent hearts per group are shown. Scale bar = 30 μ M.

(G) Representative Western blot of LC3B-I, LC3B-II, p62, and GAPDH in hearts of 20 week-old *Lmna*^{H222P/H222P} mice treated with DMSO or selumetinib (top). Numbers on top of blots denote individual heart samples. Quantification of LC3B-II and p62 normalized to α -tubulin presented as fold-change over DMSO (bottom); n = 6.

(H) Schematic of the proposed pathogenesis of *LMNA* cardiomyopathy. See text for details.