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Information about the manuscript

MKK6 deficiency promotes cardiac dysfunction through MKK3-p38 γ / δ -mTOR hyperactivation

Tracking no: 03-11-2021-RA-eLife-75250

Competing interests: No competing interests declared

Author contributions:

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Data Availability:

All data generated or analysed during this study are included in the manuscript and supporting file; Source Data files have been provided for Figures 1-7
N/A

Ethics:

Human Subjects: No Animal Subjects: Yes Ethics Statement: This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal procedures conformed to EU Directive

86/609/EEC and Recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes, enacted under Spanish law 1201/2005. All of the animals were handled according to approved institutional animal care and use committee protocols (PROEX-215/18) of the Comunidad de Madrid.

MKK6 deficiency promotes cardiac dysfunction through MKK3-p38 γ / δ -mTOR hyperactivation

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Keywords: aging/cardiac hypertrophy/MKK3/MKK6/mTOR/p38 MAPK

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ABSTRACT

Stress-activated p38 kinases control a plethora of functions and their dysregulation has been linked to development of steatosis, obesity, immune disorders and cancer. Therefore, they have been identified as potential targets for novel therapeutic strategies. There are four p38 family members (p38 α , p38 β , p38 γ , and p38 δ) that are activated by MKK3 and MKK6. Here we demonstrate that lack of MKK6 reduces the life span in mice. Longitudinal study of cardiac function in *Mkk6*^{-/-} mice showed that young mice have cardiac hypertrophy which progresses to cardiac dilatation and fibrosis with age. Mechanistically, lack of MKK6 blunts p38 α activation while causing MKK3-p38 γ/δ hyperphosphorylation and increased mTOR signaling, resulting in cardiac hypertrophy. Cardiac hypertrophy in *Mkk6*^{-/-} mice is reverted by knocking out either p38 γ or p38 δ , or by inhibiting mTOR pathway with rapamycin. In conclusion, we have identified a key role for the MKK3/6-p38 γ/δ pathway in the development of cardiac hypertrophy, which has important implications for the clinical use of p38 α inhibitors in the long-term treatment since they might result in cardiotoxicity.

INTRODUCTION

Cardiac hypertrophy is an adaptive response of the heart to hemodynamic stress that can be physiologic (e.g., pregnancy or exercise) or pathological (e.g., hypertension or valvular disease). Physiological cardiac hypertrophy is accompanied with a normal or even enhanced cardiac function, while pathological forms of hypertrophy are accompanied by myocardial dysfunction and fibrosis and represent a risk factor for ventricular arrhythmias and sudden cardiac death (Maillet *et al*, 2013; Nakamura & Sadoshima, 2018; Oldfield *et al*, 2020).

Initially, cardiac hypertrophy is induced as a compensatory response to preserve cardiac function under stressful conditions, a process known as adaptive cardiac hypertrophy. However, if the pathological stimulus is maintained, this adaptive cardiac hypertrophy will eventually lead to the development of pathological cardiac hypertrophy and heart failure (Nakamura & Sadoshima, 2018; Oldfield *et al*, 2020). The form of cardiac hypertrophy developed will depend on the type of the hypertrophic stimuli, the duration of the stimuli and the downstream signaling involved (Nakamura & Sadoshima, 2018; Oldfield *et al*, 2020; Shimizu & Minamino, 2016). Several signalling pathways known to promote physiological cardiac hypertrophic growth, when persistently activated have been found to drive pathological hypertrophy and cardiac dysfunction (Heineke & Molkentin, 2006; Maillet *et al*, 2013; Nakamura & Sadoshima, 2018; Porrello *et al*, 2008). For instance, IGF1 or Akt transgenic mice develop proportionately enlarged hearts with initially normal cardiac function, which over time progress to pathological hypertrophy with impaired cardiac function (Delaughter *et al*, 1999; Shiojima *et al*, 2005).

Stress-inducing stimuli in the heart activate several mitogen-activated protein kinases (MAPKs) including the p38 family. p38 kinases control a wide range of

processes and their dysregulation has been linked to numerous diseases, making them a promising pharmacological target for therapeutic use (Canovas & Nebreda, 2021). This family consists of four isoforms: α , β , γ , and δ , with p38 α having been the most broadly studied, whereas knowledge of the other p38 isoforms has been limited by a reduced availability of isoform-specific reagents. Our previous work showed that p38 γ and p38 δ are expressed in the heart and participate in the cardiac hypertrophic response. We have shown that p38 γ/δ mediate early postnatal cardiac hypertrophy by promoting mTOR-induced cell growth (Gonzalez-Teran *et al*, 2016).

An essential feature of both physiological and pathological hypertrophy is increased protein synthesis, critically regulated by the mammalian target of rapamycin (mTOR) pathway mainly through the phosphorylation of its downstream substrates. Activation of mTOR signaling is increased during postnatal cardiac development (Gonzalez-Teran *et al.*, 2016) as well as in the hearts of transgenic mouse models suffering from physiological cardiac hypertrophy (McMullen *et al*, 2004a; McMullen *et al*, 2004b; Shioi *et al*, 2000; Shioi *et al*, 2003). Moreover, the specific mTOR inhibitor rapamycin attenuates and reverses cardiac-overload-induced pathological hypertrophy (Shioi *et al.*, 2003). Conversely, mTOR pathway activation mediated by p38 γ and p38 δ MAPKs has been implicated in the control of postnatal cardiac hypertrophic growth and angiotensin-II-induced cardiac hypertrophy (Gonzalez-Teran *et al.*, 2016).

Here, we demonstrate that in the heart MKK3 activates p38 γ/δ , whereas MKK6 activates p38 α . Furthermore, we find that *Mkk6*^{-/-} mice exhibit cardiac hypertrophy caused by hyperactivation of the MKK3-p38 γ/δ axis, which progresses to a pathological cardiac hypertrophy phenotype with age. Our results have important implications for the clinical use of p38 α inhibitors in the long-term treatment since they might result in cardiotoxicity.

RESULTS

MKK6-deficient mice die prematurely

Several studies have addressed the role of p38 signaling in homeostasis and disease (Nikolic *et al*, 2020; Romero-Becerra *et al*, 2020). While mice lacking both MKK3 and MKK6 die in mid-gestation with mutant embryos demonstrating abnormalities of the placenta and embryonic vasculature (Brancho *et al*, 2003), mice individually lacking MKK3 or MKK6 are viable and fertile, suggesting partial functional redundancy (Lu *et al*, 1999; Tanaka *et al*, 2002; Wysk *et al*, 1999). However, the role of the p38 pathway in aging remains incompletely understood. Therefore, we examined mice harboring germline deletion of *Mkk6* (*Mkk6*^{-/-}) (Tanaka *et al*, 2002) at advanced age. Mice lacking *MKK6* have reduced body weight compared to age-matched wild type (WT) animals (Figure 1A, B), which can be partially explained by a dramatic reduction in white adipose tissue (Figure 1C). This agrees with previous studies demonstrating that *Mkk6*^{-/-} mice are protected against diet-induced obesity with increase browning of the epididymal white adipose tissue (eWAT) (Matesanz *et al*, 2017).

Additionally, these mice exhibit an abnormal posture characterized by a hunched position and the development of thoracic kyphosis and severe ataxia (Figure 1D and Figure 1 – video supplement 1). As a consequence of all these phenotypic alterations, *Mkk6*^{-/-} mice suffer premature death, the first mice dying at 51 weeks of age with a median life span of 76 weeks (Figure 1E).

MKK6-deficient mice develop increased age-related cardiac dysfunction

The downstream kinases of MKK6 have been implicated in major cardiovascular abnormalities during development. Combined deletion of p38α

and p38 β results in cardiac defects during embryonic development (del Barco Barrantes *et al.*, 2011), whereas p38 γ/δ deficient mice exhibit reduced cardiomyocyte hypertrophic growth and smaller hearts (Gonzalez-Teran *et al.*, 2016). This prompted us to speculate that cardiac abnormalities could be one of the underlying causes of premature death of *Mkk6*^{-/-} mice. Echocardiographic analyses of 12 to 14-month-old mice demonstrated eccentric hypertrophy in *Mkk6*^{-/-} mice compared to control mice, as detected by thinning of the left ventricle (LV) wall, as well as increased left ventricular internal diameter (LVID) and left ventricular volume, especially during the systole (Figure 2A). Cardiac enlargement compromised systolic function, evidenced by a decreased in the ejection fraction and fractional shortening. However, the diastolic function appeared to be maintained, with a normal E/A wave velocities ratio and isovolumetric relaxation time (Figure 2B). Moreover, *Mkk6*^{-/-} mice exhibit bradycardia (Figure 2C). We performed picrosirius red staining for collagen and quantified positive areas in serial histologic sections from *Mkk6*^{-/-} and WT hearts and found cardiac fibrotic lesions in *Mkk6*^{-/-} old mice (Figure 2D). To discard hypertension as a possible contributor of the cardiac dysfunction we evaluated blood pressure in these animals. *Mkk6*^{-/-} mice did not present differences in blood pressure compared to age-matched controls (Figure 2E).

Young MKK6-deficient mice present cardiac hypertrophy

Cardiac dysfunction may result from an initial compensated cardiac hypertrophy that with time becomes pathological (Nakamura & Sadoshima, 2018). Echocardiographic analysis at 9 weeks of age demonstrated cardiac hypertrophy in MKK6-deficient animals when compared with controls, as detected by measures of left ventricular mass, interventricular septal thickness, left ventricular posterior wall

thickness, and left ventricular internal diameter (Figure 3A). However, cardiac enlargement did not compromise systolic function or diastolic function (given by the ejection fraction and the E/A wave velocity ratio, respectively) but was accompanied by increases in stroke volume and cardiac output (Figure 3B). Gross anatomic and histologic analyses confirmed these non-invasive findings as MKK6-deficient hearts were larger than WT controls when normalized to tibia length (TL) (Figure 3C&D), a difference that was not apparent at 4 weeks of age. Serial analysis of heart weight (HW)/TL over 15 weeks demonstrated enhanced cardiac growth in *Mkk6*^{-/-} mice (Figure 3D). The progressive increase in size of *Mkk6*^{-/-} hearts correlated with increased cardiomyocyte cross-sectional area, consistent with enhanced hypertrophic growth (Figure 3E). Hypertension was excluded as a possible contributor to this increased growth as *Mkk6*^{-/-} mice demonstrate reduced systolic blood pressures when compared to age-matched controls (Figure 3 – figure supplement 1A). Fibrosis and reactivation of a “fetal gene program” (e.g., *Nppa*, *Nppb*, *Acta2*, *Myh7*) are hallmark features of pathologic hypertrophy (Bernardo *et al*, 2010). Histological cardiac examination revealed no evidence of fibrosis in *Mkk6*^{-/-} heart sections (Figure 3 – figure supplement 1B). We also found no difference in the expression of fibrotic genes including *Col1a1*, *Col3a1*, and *Fn* or markers of the fetal gene program (Figure 3 – figure supplement 1C, D). No meaningful changes were visible for *Nppa* or *Nppb* (Figure 3 – figure supplement 1D). These observations suggest that at 9 weeks of age *Mkk6*^{-/-} hearts show a non-pathological cardiac hypertrophy.

To confirm the MKK6 autonomous effect in cardiomyocytes we employed a murine conditional MKK6 allele (*Mkk6*^{LoxP})²³ and two cardiomyocyte Cre-expressing lines (*MCK-Cre* (Bruning *et al*, 1998) and *αMHC-Cre* (McFadden *et al*, 2005)) to assess the consequences of MKK6 genetic ablation in postnatal cardiomyocytes. MCK-

Cre is active in striated muscle, and the *MCK-Cre*, *Mkk6*^{MCK-KO} (*Mkk6*^{MCK-KO}) mice

demonstrated specific and efficient deletion of MKK6 in the heart and skeletal muscle tissues, but not in spleen or liver (Figure 4 – figure supplement 1A, B). Importantly, the cardiac phenotype of 9-weeks-old *Mkk6*^{MCK-KO} mice resembled that of *Mkk6*^{-/-} animals (Figure 4A-C). Similar results were obtained with *αMHC-Cre*, *Mkk6*^{LoxP/LoxP} (*Mkk6*^{αMHC-KO}) mice (Figure 4D-F). These data collectively confirm that cardiomyocyte MKK6 controls heart growth.

MKK6-deficient hearts have increased MKK3-p38γ/δ activation

MKK6 is a critical upstream activator of p38 MAPKs, but its specificity for individual p38 family isoforms is not well established. We assessed the relative levels of phosphorylated p38 isoforms by immunoprecipitation in *Mkk6*^{-/-} hearts, which demonstrated hyperphosphorylation of p38γ and p38δ (Figure 5A) with a simultaneous reduction in phosphorylation of p38α (Figure 5B). Immunoblot analysis also revealed increased levels of phosphorylated MKK3, the other main p38 upstream activator, in *Mkk6*^{-/-} hearts (Figure 5C). This observation suggested that increased phosphorylation of p38γ and p38δ in the *Mkk6*^{-/-} hearts resulted from MKK3 activation. Accordingly, phosphorylation of p38γ and p38δ was strongly reduced in MKK3-deficient mice, whereas p38α phosphorylation was not changed appreciably (Figure 5D). In addition, hearts of *Mkk3*^{-/-} mice were smaller at 9 weeks of age when compared with age-matched WT controls (Figure 5E), a finding consistent with the previously described roles of p38γ and p38δ in promoting postnatal cardiac hypertrophic growth (Gonzalez-Teran *et al.*, 2016). Taken together, these observations indicate that MKK6 primarily targets p38α and MKK3 p38γ and p38δ. In addition, they suggest that the MKK6 deficiency leads to cardiac hypertrophy via activation of MKK3 and p38γ and p38δ.

195 **Hypertrophy in MKK6-deficient hearts is mediated by p38 γ/δ**

196 To confirm that enhanced hypertrophic growth in MKK6-deficient mice is
 197 mediated by modulation of p38 γ/δ activation, we introduced a deletion of p38 γ in the
 198 context of MKK6 deficiency. The double mutant combination (*Mkk6*^{-/-}; p38 γ ^{-/-}) rescued
 199 normal cardiac growth, as the heart sizes and cardiomyocyte cross-sectional areas of the
 200 double-mutants were equivalent to those of WT controls (Figure 6A-C). We further
 201 demonstrated the requirement for p38 δ to be cell-autonomous in striated muscle using a
 202 p38 δ conditional allele (*Mapk13*^{LoxP}). MKK6-deficient hearts lacking p38 δ in their
 203 myocytes (*Mkk6*^{-/-}; *MCK-Cre*; *p38 δ* ^{LoxP/LoxP} hearts) were similar in size to those of mice
 204 lacking p38 δ in their myocytes (*MCK-Cre*; *p38 δ* ^{LoxP/LoxP} (p38 δ ^{MCK-KO})), with no
 205 appreciable increase in cardiomyocyte cross-sectional area (Figure 6D-F). Collectively,
 206 these data demonstrate that enhanced cardiac hypertrophic growth in MKK6-deficient
 207 mice is mediated by hyperactivation p38 γ/δ of signaling in striated muscle.

208

209 **mTOR pathway hyperactivation mediated hypertrophy in MKK6-deficient hearts**

210 The p38 γ and p38 δ isoforms have previously been demonstrated to promote
 211 cardiac hypertrophic growth through activation of mTOR signaling (Gonzalez-Teran *et*
 212 *al.*, 2016). As expected from that finding, immunoblot analysis of *Mkk6*^{-/-} mouse hearts
 213 showed an increase in mTOR pathway activation (Figure 7A). As protein synthesis
 214 represents a key target of the mTOR signaling pathway and is critical for cardiomyocyte
 215 hypertrophic growth, we assessed eukaryotic initiation/elongation factors by
 216 immunoblot analyses. We found an overall increase in translational activation in *Mkk6*^{-/-}
 217 hearts relative to WT (Figure 7B). We corroborated these findings by analyzing
 218 puromycin incorporation into newly synthesized peptides in WT and *Mkk6*^{-/-} hearts,

which demonstrated greater puromycin-labeling (Figure 7C) and overall protein content (Figure 7D) in mutant hearts.

We next examined the extent to which cardiac hypertrophy in *Mkk6*^{-/-} mice is mediated by increased mTOR signaling. We blocked mTOR activation by daily intraperitoneal injection of rapamycin, a potent and specific small molecule mTOR inhibitor, from 3 to 9 weeks of age. This treatment was sufficient to bring the heart size (HW/TL) of *Mkk6*^{-/-} animals close to that of WT age-matched controls, which corresponded with a robust reduction in cardiomyocyte cross-sectional area in *Mkk6*^{-/-} mice (Figure 4), altogether suggesting that the cardiac hypertrophy in *Mkk6*^{-/-} mice results from hyperactivation of mTOR signaling.

DISCUSSION

The present study provides several independent lines of evidence supporting a critical role for the MKK3/6–p38γ/δ signaling pathway in the development of cardiac hypertrophy. The hypertrophy developed seems to be physiological in young animals, with normal or even increased cardiac function at baseline. However, aging induced the development of cardiac dysfunction and premature death. Extensive analysis with different knockout mouse models shows that in the absence of MKK6, the MKK3-stimulated p38γ/δ kinases become hyperactivated and induce enhanced postnatal hypertrophic growth through the mTOR pathway. Our results also confirm that the two main up-stream p38 activators are strongly biased toward the activation of specific p38 MAPK isoforms in the heart, with p38γ/δ mainly regulated by MKK3 and p38α by MKK6, at least in homeostatic conditions.

Previous work has implicated multiple p38 isoforms in disease models of pathologic hypertrophy (Nikolic *et al.*, 2020; Romero-Becerra *et al.*, 2020), whereas

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244 p38 γ and p38 δ isoforms appeared to be involved primarily in regulating postnatal
 245 physiological cardiac growth and the metabolic switch during cardiac early postnatal
 246 development (Gonzalez-Teran *et al.*, 2016). Such reports, however, have not addressed
 247 how different p38 MAPK isoforms are regulated and the relative contributions of
 248 MKK3 and MKK6 to cardiac hypertrophic phenotypes have so far remained unclear.
 249 Indeed, either overexpression of constitutively active or dominant negative forms of
 250 MKK3/6 *in vitro* yields cardiac hypertrophy (Braz *et al.*, 2003; Streicher *et al.*, 2010;
 251 Zechner *et al.*, 1997). Our *in vivo* data support a model wherein MKK3 activity
 252 promotes hypertrophic growth. We show that MKK3 hyperactivation resulting from
 253 MKK6 deletion leads to cardiac hypertrophy while MKK3 genetic ablation results in
 254 reduced postnatal cardiac growth. We also demonstrate that deficiency of MKK6 in
 255 cardiomyocytes is sufficient to result in cardiac hypertrophy, attributable to both,
 256 MKK6 direct function in inducing hypertrophy via p38-mediated signaling as well as its
 257 role as a negative regulator of MKK3 activity. This negative regulation may be a direct
 258 action of MKK6 or may be mediated by p38 α , whose phosphorylation levels are
 259 strongly decreased in *Mkk6*^{-/-} hearts. The last possibility is further supported by evidence
 260 indicating that p38 α might negatively regulate the MKK3/6 upstream kinase MAP3K
 261 TAK1 (Singhirunnusorn *et al.*, 2005). Indeed, we have found that in other tissues, lack
 262 of p38 α induces p38 γ and p38 δ activation (Matesanz *et al.*, 2018). The negative
 263 feedback activity of p38 α is consistent with MKK3 hyperphosphorylation identified in
 264 *Mkk6*^{-/-} mice. Our results provide the first demonstration that MKK3 preferentially
 265 regulates p38 γ and p38 δ activation, whereas MKK6 is responsible for p38 α regulation.
 266 MKK6-deficient mice show p38 γ/δ hyperactivation and impaired p38 α
 267 activation, possibly implicating any of these isoforms in the observed phenotypes.
 268 Through the use of multiple *in vivo* models, we show that hyperphosphorylated MKK3

promotes cardiac hypertrophic growth through activation of p38 γ - and p38 δ -mediated

mTOR signaling in the heart. This is consistent with our earlier work showing an essential role for these p38 isoforms in mTOR-dependent of physiologic and pathological cardiac hypertrophy (Gonzalez-Teran *et al.*, 2016), as well as with previous reports indicating that p38 α does not mediate hypertrophic responses in animal models of pressure-overload cardiac hypertrophy (Nishida *et al.*, 2004). This conclusion is corroborated by the reversion of cardiac hypertrophy in MKK6-deficient mice also deficient for cardiac p38 γ or p38 δ , and further corroboration comes from the ability of the mTOR inhibitor rapamycin to prevent cardiac hypertrophy during early postnatal cardiac development in MKK6-deficient mice. These findings correlate with impaired p38 γ and p38 δ activation and reduced postnatal hypertrophic growth in MKK3-deficient mice.

The p38 γ - and p38 δ -mediated control of cardiac hypertrophy during postnatal cardiac development resides in cardiomyocytes (Gonzalez-Teran *et al.*, 2016). Accordingly, lack of MKK6 in skeletal muscle or cardiomyocytes yields the same phenotype as global MKK6 deficiency. Furthermore, the blockade of cardiac hypertrophy in MKK6-deficient mice upon deletion of p38 δ specifically in striated muscle indicates that cardiac p38 δ lies downstream of MKK6 in the signaling pathway controlling hypertrophic growth. Interestingly, reversion of the MKK6-deficient phenotype upon p38 δ deletion was greater than that achieved upon deletion of p38 γ (Figure 6), consistent with the reported dominance of p38 δ in regulating cardiac hypertrophic growth (Gonzalez-Teran *et al.*, 2016).

Young MKK6-deficient mice develop a cardiac hypertrophy that could be classified as physiological, characterized by a proportionate increase in heart size with maintenance of a normal cardiac structure. Moreover, cardiac function was normal, with

294 increased cardiac output and stroke volume, and there was no evidence of fibrosis nor
 295 re-expression of the cardiac stress fetal gene program. Physiologic cardiac hypertrophy
 296 is an adaptive response that increases ventricular mass while maintaining or enhancing
 297 cardiac function (Kang, 2006; Nakamura & Sadoshima, 2018). However, it has
 298 increasingly become appreciated that sustained activation of the pathways that drive this
 299 beneficial response can ultimately result in pathological remodeling and associated
 300 sudden cardiac death (Condorelli *et al*, 2002; Lauschke & Maisch, 2009; Matsui *et al*,
 301 2002; McMullen *et al.*, 2004a; Oldfield *et al.*, 2020; Shioi *et al*, 2002). In agreement
 302 with this, the cardiac hypertrophy observed in *Mkk6*^{-/-} mice becomes deleterious with
 303 age, compromising the cardiac function and likely contributing to the reduced survival
 304 observed in these mice. Our results identify a key role for the MKK3/6-p38γ/δ pathway
 305 in the development of cardiac hypertrophy and illustrates how, depending on the
 306 stimulus, the activation of the same pathway can promote the progression from a
 307 physiological to a pathological phenotype.

308 The activation of p38α pathway has been linked to several diseases, suggesting
 309 that this pathway could represent a target for their treatment. However, the results from
 310 the clinical trials have been disappointing so far (Canovas & Nebreda, 2021). p38α
 311 inhibitors have been the more profoundly studied. However, these studies usually do not
 312 consider the possible undesired effect of p38α inhibition upon the other p38 pathway
 313 kinases, which could be among the reasons of failure in the outcomes. Our results show
 314 an example of how p38α inhibition leads to an unexpected activation of MKK3-p38γ/δ,
 315 having deleterious effects in the heart in the long-term. Our finding suggests that
 316 treatment strategies using longstanding p38α inhibition should consider the potential
 317 cardiovascular risk among the possible secondary effects of the treatment.

318

MATERIALS AND METHODS

Animal preparation

Mkk3^{-/-} mice (B6.129-*Map2k3*^{tm1Flv}) (Lu *et al.*, 1999; Wysk *et al.*, 1999) and *Mkk6*^{-/-} mice (B6.129-*Map2k6*^{tm1Flv}) (Brancho *et al.*, 2003) were as previously described. Mice with a germ-line mutation in the *Map2k6* gene and *LoxP* elements inserted into two introns (*Map2k6*^{LoxP}) were generated as previously described²⁴. To generate mice lacking MKK6 or p38δ in striated muscle, *Map2k6*^{LoxP} or p38δ-negative (B6.129-Mapk13tm1) mice were crossed with the FVB-Tg(Ckmm-cre)5Khn/J line on the C57BL/6J background (Jackson Laboratory). Mice lacking MKK6 in cardiomyocytes were generated by crossing *Map2k6*^{LoxP} mice with the Tg(Myh6-cre)2182Mds line on the C57BL/6J background (Jackson Laboratory). The p38γ-negative line (B6.129-Mapk12tm1) was crossed with the *Mkk6*^{-/-} line (B6.129-*Map2k6*^{tm1Flv}) to generate double knockout mice. Likewise, mice lacking p38δ in striated muscle were crossed with the *Mkk6*^{-/-} (B6.129-*Map2k6*^{tm1Flv}) line. Genotype was confirmed by PCR analysis of genomic DNA. For signaling studies, animals were killed by cervical dislocation. For rapamycin treatment, mice received daily intraperitoneal injections with rapamycin (LC Laboratories, R-5000) (2 mg kg⁻¹ per day) or vehicle (0.25 % polyethylene glycol (Sigma), 0.25 % Tween-20 (Sigma) in PBS); injections started at 4 weeks of age and continued until 9 weeks of age, when heart size was analyzed by echocardiography. All animal procedures conformed to EU Directive 86/609/EEC and Recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes, enacted under Spanish law 1201/2005.

Computed tomography scan

343 Computed tomography (CT) studies were performed with a small-animal PET/CT
344 scanner (nanoScan, Mediso, Hungary). For the acquisition, mice were anesthetized
345 using isoflurane 2% and 1.8 L/min oxygen flow. Ophthalmic gel was placed in the eyes
346 to prevent drying. CT was acquired using an X-ray beam current of 178 μ A and a tube
347 voltage of 55 kVp with 360 projections of 500 ms in a helical scan with pitch 1 and
348 binning 1:4. CT image was reconstructed using a Ramlack algorithm with a final
349 resolution of 0.078 mm³.

351 Histology

352 Tissue samples were fixed in 10% formalin for 48 h, dehydrated, and embedded in
353 paraffin. Sections (8 μ m) were cut and stained with hematoxylin and eosin (American
354 Master Tech Scientific). Fibrosis was assessed by Picrosirius red staining (Sigma) and
355 the positive area for fibrosis was quantified with Image J software(Schneider *et al*,
356 2012). For wheat germ agglutinin (WGA) immunofluorescence, 8 μ m heart sections
357 were prepared, washed in PBS, incubated for 2h in WGA-Alexa 488 lectin (Invitrogen,
358 Carlsbad, CA, USA), and washed and mounted in anti-fade reagent. Four images ($\times$ 20)
359 were taken from each heart, and the areas of 100–200 cross-sectionally oriented
360 cardiomyocytes were measured and analyzed with Image J software(Schneider *et al*,
361 2012).

363 Echocardiography

364 Mice were anesthetized by inhalation of isoflurane and oxygen (1.25 % and 98.75 %,
365 respectively), and echocardiography was performed with a 30-MHz transthoracic
366 echocardiography probe. Images were obtained with the Vevo 2100 micro-ultrasound
367 imaging system (VisualSonics, Toronto, Canada). Short-axis, long-axis, B-mode and

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two-dimensional M-mode views were obtained. Scans were conducted by two experienced researchers blinded to the mouse genotype. Measurements of left parasternal long and short axes and M-mode images (left parasternal short axis) were obtained at a heart rate of 500–550 b.p.m. LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), and wall thickness were measured from M-mode tracings, and the average of three consecutive cardiac cycles is reported. The LV fractional shortening percentage was calculated as $([LVEDD - LVESD] / LVEDD) \times 100$. MRI of lung was performed with a 7-T Agilent scanner (Agilent, Santa Clara, CA, USA) equipped with a DD2 console and an actively shielded gradient set (205/120 insert of maximum 130 mT m⁻¹ gradient strength). To enhance the signal-to-noise ratio during image acquisition, we used a combination of a 72-mm inner diameter quadrature birdcage TX volume coil (Rapid Biomedical GmbH, Germany) and an actively detuning 30-mm flexible customized surface RX coil (Neos Biotec, Pamplona, Spain). After acquisition of a tripilot gradient-echo image, a gradient-echo sequence without gating was used to acquire oblique coronal slices (1-2 slices) and axial slices (7-10 slices covering the entire lung, 72-s acquisition time per slice) using the following parameters: TR/TE=6.7/2.2 ms, flip angle=10 degree, bandwidth=100 kHz, field of view=3 × 3 cm, matrix=256 × 128, slice thickness=1 mm (ref. 40). These images were used to determine interventricular septum and left ventricle posterior wall thicknesses and left ventricle corrected mass; the short-axis M-mode quantification was chosen as the most representative. Function was estimated from the ejection fraction and fractional shortening obtained from M-mode views by a blinded echocardiography expert. For ejection fraction measurements, a long- or short-axis view of the heart was selected to obtain an M-mode registration in a line perpendicular to the left ventricular septum and posterior wall at the level of the mitral chordae tendineae.

394 Immunoblot analysis

395 Tissue extracts were prepared in Triton (20 mM Tris (pH 7.4), 1 % Triton X-100, 10 %
 396 glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β -glycerophosphate, 1 mM sodium
 397 orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 $\mu\text{g ml}^{-1}$ aprotinin and
 398 leupeptin). Extracts (20–50 μg protein) and immunoprecipitates (prepared from 0.5-2
 399 mg) were examined by immunoblot. For immunoprecipitation assays, heart extracts
 400 were incubated with 1-4 μg of a specific antibody coupled to protein-G-Sepharose.
 401 After incubation overnight at 4 °C with agitation, the captured proteins were centrifuged
 402 at 10,000g, the supernatants collected, and the beads washed four times in PBS1X.
 403 Beads were boiled for 5 min at 95 °C in 10 μl sample buffer. Extracts and
 404 immunoprecipitates were examined by SDS–PAGE and blotted with antibodies to the
 405 following targets: p38 γ and p38 δ (Sabio *et al*, 2005; Sabio *et al*, 2004) at 1 $\mu\text{g ml}^{-1}$;
 406 vinculin (Sigma); puromycin (Millipore clone 12D10); phospho-MKK3
 407 (Ser189)/MKK6 (Ser207), MKK3, MKK6, phospho-p38 MAPK (Thr180/Tyr182),
 408 phospho-mTOR (Ser2481), mTOR, phospho-p70S6 kinase (Thr 389) (108D2), p70S6
 409 kinase, phospho-S6 (Ser 235/236) (D57.2.2E), phospho-S6 (Ser 240/244) (61H9), S6
 410 ribosomal protein, phospho-FoxO1 (Thr24)/FoxO3a (Thr32), phospho-eEF2 (Thr56),
 411 phospho-eIF4G (Ser1108), phospho-eIF4B (Ser422), eIF4B, phospho-4EBP1
 412 (Thr37/46), and 4EBP1, all at a 1:1000 dilution. Immunocomplexes were detected by
 413 enhanced chemiluminescence (GE Healthcare Lifesciences).

414

415 *In vivo* protein synthesis assay

416 For all *in vivo* measurements of protein synthesis, mice were injected intraperitoneally
 417 with 0.040 $\mu\text{mol g}^{-1}$ puromycin dissolved in 100 μl PBS. Exactly 30 min after injection,

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tissues were extracted and frozen in liquid N₂ for subsequent immunoblot analysis of protein-incorporated puromycin.

Blood pressure and heart rate measurements

Blood pressure and heart rate in mice was measured using the noninvasive tail-cuff method(Kubota *et al*, 2006). The measures were performed in conscious mice placed in a BP-2000 Blood Pressure Analysis System (Visitech Systems). 10 preliminary measurements and 10 actual measurements were recorded and the average of the 10 actual measurements used for analysis. The animals were trained for 4 consecutive days prior the actual measurements were registered. All the measurements were taken at the same time of the day.

RT-qPCR

RNA 500ng – extracted with RNAeasy Plus Mini kit (Qiagen) following manufacturer instructions – was transcribed to cDNA, and RT-qPCR was performed using Fast Sybr Green probe (Applied Biosystems) and the appropriate primers in the 7900 Fast Real Time thermocycler (Applied Biosystems). Relative mRNA expression was normalized to *Gapdh* mRNA measured in each sample. *Fnl* Fw: ATGTGGACCCCTCCTGATAGT, Rev: GCCCAGTGATTTCAGCAAAGG; *Coll1a1* Fw: GCTCCTCTTAGGGGCCACT, Rev: CCACGTCTCACCATTGGGG; *Col3a1* Fw: CTGTAACATGGAAACTGGGGAAA, Rev: CCATAGCTGAACTGAAAACCACC; *Nppa* Fw: GCTTCCAGGCCATATTGGAG, Rev: GGGGGCATGACCTCATCTT; *Nppb* Fw: GAGGTCACCTATCCTCTGG, Rev: GCCATTTCTCCTCCGACTTTTCTC; *Acta-2* Fw: CCCAAAGCTAACCGGGAGAAG, Rev: CCAGAATCCAACACGATGCC; *Myh7* Fw: ACTGTCAACACTAAGAGGGTCA, Rev:

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TTGGATGATTTGATCTTCCAGGG; *Gapdh* FW: TGAAGCAGGCATCTGAGGG, Rev:

CGAAGGTGGAAGAGTGGGA

Statistical analysis

Results are expressed as mean $\pm$ SEM. A difference of $P < 0.05$ was considered significant. Gaussian (normal) distribution was determined using the Shapiro-Wilks normality test. For normally distributed populations, differences between groups were examined for statistical significance by two-tailed Student *t*-test (2 groups) and 1-way ANOVA followed by Tukey post-test (3 or more groups). To test the respective roles of treatment or age and genotype, a 2-way ANOVA was performed. Tukey or Sidak post-test were subsequently employed when appropriate. For data that failed normality testing, Mann-Whitney test (2 groups), or Kruskal-Wallis with Dunn post-test (3 or more groups) was performed. Gehan-Breslow-Wilcoxon test was used to assess significance in the Kaplan–Meier survival analysis.

ACKNOWLEDGEMENTS

We thank S. Bartlett and F. Chanut for English editing. We are grateful to R.J. Davis, A. Padmanabhan, M. Costa and C. López-Otín for critical reading of the manuscript. We thank A. C. Silva (ana@anasilvaillustrations.com) for help with figure editing and design. This work was funded by a CNIC Intramural Project Severo Ochoa (Expediente 12-2016 IGP) to GS and JJ and MINECO-FEDER, PID2019-104399RB-I00 to GS. BGT was a fellow of FPI Severo Ochoa CNIC Program (SVP-2013-067639) and is an American Heart Association Postdoctoral Fellow (18POST34080175). R.R.B is a fellow of the FPU Program (FPU17/03847). The following grants provided additional funding: GS is granted by funds from European Regional Development Fund (ERDF):

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EFSD/Lilly European Diabetes Research Programme Dr Sabio, Fundación AECC

PROYE19047SABI and Comunidad de Madrid IMMUNOTHERCAN-CM

B2017/BMD-3733; US National Heart, Lung, and Blood Institute (R01 Grant

HL122352), Fondos FEDER, Madrid, Spain, and Fundación Bancaria “La Caixa

(project HR19/52160013); Fundación La Marató TV3: Ayudas a la investigación en

enfermedades raras 2020 (LA MARATO-2020); and Instituto de Salud Carlos III to JJ.

IN. was funded by EFSD/Lilly grants (2017 and 2019), the CNIC IPP FP7 Marie Curie

Programme (PCOFUND-2012-600396), EFSD Rising Star award (2019), JDC-2018-

Incorporación (MIN/JDC1802). The CNIC is supported by the Instituto de Salud Carlos

III (ISCIH), the Ministerio de Ciencia e Innovación (MCIN) and the Pro CNIC

Foundation)

AUTHOR CONTRIBUTIONS

G.S. conceived this project. B.G-T. and R.R-B. equally performed the primary

experiments, acquired, prepared figures, and analyzed the data. G.S., B.G-T. and R.R-B.

designed, developed the hypothesis and wrote the manuscript with input from all authors.

B.G-T., R.R-B. A.M., E.M., L.S., I.N., V.M-R., F.M.C.U., A.M.S., M.E.R., L.L-V.,

L.J.J-B, J.J., D.F-R. and V.B. help to perform some of the experiments and acquired

data and critically revised the manuscript. G.S. led and funded the project.

COMPETING INTEREST

The authors declare that they have no competing interest.

REFERENCES

Bernardo BC, Weeks KL, Pretorius L, McMullen JR (2010) Molecular distinction between physiological and pathological cardiac hypertrophy: experimental findings and therapeutic strategies. *Pharmacol Ther* 128: 191-227

- 492 Brancho D, Tanaka N, Jaeschke A, Ventura JJ, Keikar N, Tanaka Y, Kyuuma M,
493 Takeshita T, Flavell RA, Davis RJ (2003) Mechanism of p38 MAP kinase activation in
494 vivo. *Genes Dev* 17: 1969-1978
- 495 Braz JC, Bueno OF, Liang Q, Wilkins BJ, Dai YS, Parsons S, Braunwart J, Glascock
496 BJ, Klevitsky R, Kimball TF *et al* (2003) Targeted inhibition of p38 MAPK promotes
497 hypertrophic cardiomyopathy through upregulation of calcineurin-NFAT signaling. *J*
498 *Clin Invest* 111: 1475-1486
- 499 Bruning JC, Michael MD, Winnay JN, Hayashi T, Horsch D, Accili D, Goodyear LJ,
500 Kahn CR (1998) A muscle-specific insulin receptor knockout exhibits features of the
501 metabolic syndrome of NIDDM without altering glucose tolerance. *Mol Cell* 2: 559-569
- 502 Canovas B, Nebreda AR (2021) Diversity and versatility of p38 kinase signalling in
503 health and disease. *Nat Rev Mol Cell Biol* 22: 346-366
- 504 Condorelli G, Drusco A, Stassi G, Bellacosa A, Roncarati R, Iaccarino G, Russo MA,
505 Gu Y, Dalton N, Chung C *et al* (2002) Akt induces enhanced myocardial contractility
506 and cell size in vivo in transgenic mice. *Proc Natl Acad Sci U S A* 99: 12333-12338
- 507 del Barco Barrantes I, Coya JM, Maina F, Arthur JS, Nebreda AR (2011) Genetic
508 analysis of specific and redundant roles for p38alpha and p38beta MAPKs during
509 mouse development. *Proc Natl Acad Sci U S A* 108: 12764-12769
- 510 Delaughter MC, Taffet GE, Fiorotto ML, Entman ML, Schwartz RJ (1999) Local
511 insulin-like growth factor I expression induces physiologic, then pathologic, cardiac
512 hypertrophy in transgenic mice. *FASEB J* 13: 1923-1929
- 513 Gonzalez-Teran B, Lopez JA, Rodriguez E, Leiva L, Martinez-Martinez S, Bernal JA,
514 Jimenez-Borreguero LJ, Redondo JM, Vazquez J, Sabio G (2016) p38gamma and delta
515 promote heart hypertrophy by targeting the mTOR-inhibitory protein DEPTOR for
516 degradation. *Nat Commun* 7: 10477
- 517 Heineke J, Molkentin JD (2006) Regulation of cardiac hypertrophy by intracellular
518 signalling pathways. *Nat Rev Mol Cell Biol* 7: 589-600
- 519 Kang YJ (2006) Cardiac hypertrophy: a risk factor for QT-prolongation and cardiac
520 sudden death. *Toxicol Pathol* 34: 58-66
- 521 Kubota Y, Umegaki K, Kagota S, Tanaka N, Nakamura K, Kunitomo M, Shinozuka K
522 (2006) Evaluation of blood pressure measured by tail-cuff methods (without heating) in
523 spontaneously hypertensive rats. *Biol Pharm Bull* 29: 1756-1758
- 524 Lauschke J, Maisch B (2009) Athlete's heart or hypertrophic cardiomyopathy? *Clin Res*
525 *Cardiol* 98: 80-88
- 526 Lu HT, Yang DD, Wysk M, Gatti E, Mellman I, Davis RJ, Flavell RA (1999) Defective
527 IL-12 production in mitogen-activated protein (MAP) kinase kinase 3 (Mkk3)-deficient
528 mice. *Embo J* 18: 1845-1857
- 529 Maillet M, van Berlo JH, Molkentin JD (2013) Molecular basis of physiological heart
530 growth: fundamental concepts and new players. *Nat Rev Mol Cell Biol* 14: 38-48
- 531 Matesanz N, Bernardo E, Acin-Perez R, Manieri E, Perez-Sieira S, Hernandez-Cosido
532 L, Montalvo-Romeral V, Mora A, Rodriguez E, Leiva-Vega L *et al* (2017) MKK6
533 controls T3-mediated browning of white adipose tissue. *Nat Commun* 8: 856
- 534 Matesanz N, Nikolic I, Leiva M, Pulgarin-Alfaro M, Santamans AM, Bernardo E, Mora
535 A, Herrera-Melle L, Rodriguez E, Beiroa D *et al* (2018) p38alpha blocks brown adipose
536 tissue thermogenesis through p38delta inhibition. *PLoS Biol* 16: e2004455
- 537 Matsui T, Li L, Wu JC, Cook SA, Nagoshi T, Picard MH, Liao R, Rosenzweig A
538 (2002) Phenotypic spectrum caused by transgenic overexpression of activated Akt in the
539 heart. *J Biol Chem* 277: 22896-22901

540 McFadden DG, Barbosa AC, Richardson JA, Schneider MD, Srivastava D, Olson EN
541 (2005) The Hand1 and Hand2 transcription factors regulate expansion of the embryonic
542 cardiac ventricles in a gene dosage-dependent manner. *Development* 132: 189-201
543 McMullen JR, Shioi T, Huang WY, Zhang L, Tarnavski O, Bisping E, Schinke M,
544 Kong S, Sherwood MC, Brown J *et al* (2004a) The insulin-like growth factor 1 receptor
545 induces physiological heart growth via the phosphoinositide 3-kinase(p110alpha)
546 pathway. *J Biol Chem* 279: 4782-4793
547 McMullen JR, Shioi T, Zhang L, Tarnavski O, Sherwood MC, Dorfman AL, Longnus
548 S, Pende M, Martin KA, Blenis J *et al* (2004b) Deletion of ribosomal S6 kinases does
549 not attenuate pathological, physiological, or insulin-like growth factor 1 receptor-
550 phosphoinositide 3-kinase-induced cardiac hypertrophy. *Mol Cell Biol* 24: 6231-6240
551 Nakamura M, Sadoshima J (2018) Mechanisms of physiological and pathological
552 cardiac hypertrophy. *Nat Rev Cardiol* 15: 387-407
553 Nikolic I, Leiva M, Sabio G (2020) The role of stress kinases in metabolic disease. *Nat*
554 *Rev Endocrinol*
555 Nishida K, Yamaguchi O, Hirotani S, Hikoso S, Higuchi Y, Watanabe T, Takeda T,
556 Osuka S, Morita T, Kondoh G *et al* (2004) p38alpha mitogen-activated protein kinase
557 plays a critical role in cardiomyocyte survival but not in cardiac hypertrophic growth in
558 response to pressure overload. *Mol Cell Biol* 24: 10611-10620
559 Oldfield CJ, Duhamel TA, Dhalla NS (2020) Mechanisms for the transition from
560 physiological to pathological cardiac hypertrophy. *Can J Physiol Pharmacol* 98: 74-84
561 Porrello ER, Widdop RE, Delbridge LM (2008) Early origins of cardiac hypertrophy:
562 does cardiomyocyte attrition programme for pathological 'catch-up' growth of the heart?
563 *Clin Exp Pharmacol Physiol* 35: 1358-1364
564 Romero-Becerra R, Santamans AM, Folgueira C, Sabio G (2020) p38 MAPK Pathway
565 in the Heart: New Insights in Health and Disease. *Int J Mol Sci* 21
566 Sabio G, Arthur JS, Kuma Y, Pegg M, Carr J, Murray-Tait V, Centeno F, Goedert M,
567 Morrice NA, Cuenda A (2005) p38gamma regulates the localisation of SAP97 in the
568 cytoskeleton by modulating its interaction with GKAP. *Embo J* 24: 1134-1145
569 Sabio G, Reuver S, Feijoo C, Hasegawa M, Thomas GM, Centeno F, Kuhlendahl S,
570 Leal-Ortiz S, Goedert M, Garner C *et al* (2004) Stress- and mitogen-induced
571 phosphorylation of the synapse-associated protein SAP90/PSD-95 by activation of
572 SAPK3/p38gamma and ERK1/ERK2. *Biochem J* 380: 19-30
573 Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of
574 image analysis. *Nat Methods* 9: 671-675
575 Shimizu I, Minamino T (2016) Physiological and pathological cardiac hypertrophy. *J*
576 *Mol Cell Cardiol* 97: 245-262
577 Shioi T, Kang PM, Douglas PS, Hampe J, Yballe CM, Lawitts J, Cantley LC, Izumo S
578 (2000) The conserved phosphoinositide 3-kinase pathway determines heart size in mice.
579 *EMBO J* 19: 2537-2548
580 Shioi T, McMullen JR, Kang PM, Douglas PS, Obata T, Franke TF, Cantley LC, Izumo
581 S (2002) Akt/protein kinase B promotes organ growth in transgenic mice. *Mol Cell Biol*
582 22: 2799-2809
583 Shioi T, McMullen JR, Tarnavski O, Converso K, Sherwood MC, Manning WJ, Izumo
584 S (2003) Rapamycin attenuates load-induced cardiac hypertrophy in mice. *Circulation*
585 107: 1664-1670
586 Shiojima I, Sato K, Izumiya Y, Schiekofer S, Ito M, Liao R, Colucci WS, Walsh K
587 (2005) Disruption of coordinated cardiac hypertrophy and angiogenesis contributes to
588 the transition to heart failure. *J Clin Invest* 115: 2108-2118

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Singhirunnusorn P, Suzuki S, Kawasaki N, Saito I, Sakurai H (2005) Critical roles of threonine 187 phosphorylation in cellular stress-induced rapid and transient activation of transforming growth factor-beta-activated kinase 1 (TAK1) in a signaling complex containing TAK1-binding protein TAB1 and TAB2. *J Biol Chem* 280: 7359-7368

Streicher JM, Ren S, Herschman H, Wang Y (2010) MAPK-activated protein kinase-2 in cardiac hypertrophy and cyclooxygenase-2 regulation in heart. *Circ Res* 106: 1434-1443

Tanaka N, Kamanaka M, Enslen H, Dong C, Wisk M, Davis RJ, Flavell RA (2002) Differential involvement of p38 mitogen-activated protein kinase kinases MKK3 and MKK6 in T-cell apoptosis. *EMBO Rep* 3: 785-791

Wisk M, Yang DD, Lu HT, Flavell RA, Davis RJ (1999) Requirement of mitogen-activated protein kinase kinase 3 (MKK3) for tumor necrosis factor-induced cytokine expression. *Proc Natl Acad Sci U S A* 96: 3763-3768

Zechner D, Thuerauf DJ, Hanford DS, McDonough PM, Glembotski CC (1997) A role for the p38 mitogen-activated protein kinase pathway in myocardial cell growth, sarcomeric organization, and cardiac-specific gene expression. *J Cell Biol* 139: 115-127

FIGURES

Figure 1. *Mkk6*^{-/-} mice show a reduced survival age. (A) Representative picture of 19-month-old WT and *Mkk6*^{-/-} male mice. Scale bar: 2 cm. (B) Body weight of WT (n=10-15) and *Mkk6*^{-/-} (n=4-16) mice over the indicated age period. 2-way ANOVA followed by Sidak's post test. (C) Tibia length and body weight, subcutaneous white adipose tissue (sWAT) and epididymal white adipose tissue (eWAT) to tibia length ratio from 20-month-old WT (n=10-11) and *Mkk6*^{-/-} (n=8-10) mice. Unpaired *t*-test or Mann-Whitney test. (D) Representative CT scan images and quantification of the column kyphosis angle of 19-week-old WT (n=5) and *Mkk6*^{-/-} (n=5) mice. Unpaired *t*-test. (E)

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616 Kaplan-Meier survival plot of age-related mortality in WT ($n=30$) and *Mkk6*^{-/-} ($n=33$)
 617 mice. An endpoint of 105 weeks was chosen to avoid a severe worsening of the mice
 618 health. Gehan-Breslow-Wilcoxon test. Data in B-D are mean $\pm$ SEM. ** $P<0.01$;
 619 *** $P<0.001$.

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620 **Figure 1 – video supplement 1. Ataxia and hunched posture in *Mkk6*^{-/-} mice.** Video

621 showing the movement of 18-month-old WT (left) and *Mkk6*^{-/-} (right) mice. WT: wild

622 type.

623

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Figure 2. MKK6 deficiency promotes cardiac dysfunction at advanced ages. (A, B)

Echocardiography parameters related to left ventricle (LV) dimensions (A) and contractility (B) in 12 to 14-month-old WT (*n*=8) and *Mkk6*^{-/-} (*n*=13). Each dot corresponds to an individual animal. LV wall thickness;d (left ventricle wall thickness in diastole), LVID;d (left ventricular internal diameter in diastole), LVID;s (left ventricular internal diameter in systole), LV volume;d (left ventricular volume in diastole), LV volume;s (left ventricular volume in systole). Unpaired *t*-test. (C) Heart rate in conscious 18-month-old WT (*n*=9) and *Mkk6*^{-/-} (*n*=7) mice. bpm (beats per minute). Unpaired *t*-test. (D) Picrosirius red staining and quantification of cardiac fibrosis in 23 to 24-month-old WT (*n*=8) and *Mkk6*^{-/-} (*n*=6) mice. Mann-Whitney test. Scale bars: 250 μm. (E) Systolic and diastolic blood pressure in measured in conscious

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636 18-month-old WT ($n=9$) and $Mkkb^{-/-}$ ($n=7$) mice. Unpaired t -test. Data in A-E are mean

637 $\pm$ SEM. * $P<0.05$; ** $P<0.01$.

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Figure 3. Young MKK6-deficient hearts are hypertrophic with preserved cardiac function. (A, B) Echocardiography parameters related to left ventricle (LV) dimensions (A) and contractility (B) in 9-week-old WT (*n*=37-54) and *Mkk6*^{-/-} (*n*=29-46) mice. Each dot corresponds to an individual mouse. Mean ± SEM are shown as well. LV Mass (left ventricular mass), LVID;d (left ventricular internal diameter in diastole), LVPW;d (left ventricular posterior wall in diastole), IVS;d (inter-ventricular septum in

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645 diastole). Unpaired *t*-test or Mann-Whitney test. (C) Representative whole hearts and
646 cardiac longitudinal sections stained with hematoxylin & eosin (H&E) from 9-week-old
647 WT and *Mkk6*^{-/-} mice. Scale bars: 1 mm. (D) Heart weight to tibia length ratio (HW/TL)
648 of WT (*n*=4-15) and *Mkk6*^{-/-} (*n*=5-14) over the indicated age period. 2-way ANOVA
649 followed by Sidak's post test. (E) *Top*: Representative FITC wheat germ agglutinin
650 (FITC-WGA)-stained heart sections from 9-week-old WT and *Mkk6*^{-/-} mice and
651 quantification of cardiomyocyte cross-sectional area over time (right graph, WT *n*=4-5;
652 *Mkk6*^{-/-} *n*=4-6, 2-way ANOVA followed by Sidak's post test). Scale bars: 25 μm.
653 *Bottom*. Representative H&E-stained heart sections. Scale bars: 50 μm. Data in A, B, D
654 and E are mean ± SEM. **P*<0.05; ***P*<0.01; ****P*<0.001.

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Figure 3 – figure supplement 1. Evaluation of hallmarks of pathological cardiac hypertrophy in young $Mkk6^{-/-}$ mice. (A) Systolic blood pressure in WT ($n=5$) and $Mkk6^{-/-}$ ($n=4-7$) mice at the indicated times after birth. 2-way ANOVA followed by Sidak's post test. (B) Picrosirius red staining and quantification of cardiac fibrosis in 9-week-old WT ($n=4$) and $Mkk6^{-/-}$ ($n=3$) mice. Unpaired t -test. Scale bars: 1mm. (C-D) Cardiac gene expression of fibrosis (C) and cardiac stress (D) markers in 9-week-old WT ($n=8$) and $Mkk6^{-/-}$ ($n=8$). Unpaired t -test or Mann-Whitney test. Data in A-D are mean $\pm$ SEM. * $P<0.05$. ** $P<0.001$

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Figure 4. Cardiac MKK6 controls postnatal heart growth. (A) Heart weight to tibia length ratio (HW/TL) in 9-week-old MCK-Cre ($n=5$) and Mkk6^{MCK-KO} ($n=8$) mice.

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667 Unpaired *t*-test. **(B)** H&E-stained transverse cardiac sections from control (MCK-Cre)
668 and Mkk6^{MCK-KO} mice. Scale bars: 1 mm. **(C)** Representative FITC wheat germ
669 agglutinin (FITC-WGA) staining and corresponding quantification of cardiomyocyte
670 cross-sectional area in MCK-Cre (*n*=4) and Mkk6^{MCK-KO} (*n*=5) mice. Unpaired *t*-test.
671 Scale bars: 25 μm. **(D)** Heart weight to tibia length ratio in 9-week-old αMHC-Cre
672 (*n*=5) and MKK6^{αMHC-KO} (*n*=7) mice. Unpaired *t*-test. **(E)** H&E-stained transverse heart
673 sections from αMHC-Cre and MKK6^{αMHC-KO} mice. Scale bars: 1 mm. **(F)**
674 Representative FITC-WGA staining and corresponding quantification of cardiomyocyte
675 cross-sectional area in αMHC-Cre (*n*=6) and MKK6^{αMHC-KO} (*n*=6) mice. Unpaired *t*-
676 test. Scale bars: 25 μm. Data in A, C, D and F are mean ± SEM. **P*<0.05; ***P*<0.01.

Figure 4 – figure supplement 1. Tissue-specific MKK6 deletion in *Mkk6*^{MCK-KO} and *Mkk6* ^{α MHC-KO} mice. (A) Confirmation of MKK6 deletion in tissue lysates from *Mkk6*^{MCK-KO} (KO) mice in quadriceps (Quad), gastronemius (Gastr), soleus and heart, but not in spleen and liver, and not in MCK-Cre (WT) tissues. (B) Confirmation of specific MKK6 deletion in the heart of *MKK6* ^{α MHC-KO}, but not in lysates of α MHC-Cre (WT) and *MKK6* ^{α MHC-KO} (KO) quadriceps (Quad), kidney, spleen, brain, liver, epididymal white adipose tissue (eWAT) and brown adipose tissue (BAT).

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Figure 5. MKK6 is necessary for p38α phosphorylation and MKK3 for p38γ and p38δ phosphorylation in the heart. (A) Western blot analysis of the phosphorylation and amount of p38γ and δ immunoprecipitated from heart lysates from 9-week-old WT and *Mkk6*^{-/-} mice. (B) Immunoblot analysis of p38α phosphorylation and protein amount in WT and *Mkk6*^{-/-} mice. (C) Phosphorylation and protein levels of MKK3 and MKK6 in heart lysates from WT and *Mkk6*^{-/-} mice. (D) Immunoprecipitation analysis of the phosphorylation and protein amounts of p38α, p38γ and p38δ isoforms in heart lysates from 9-week-old WT and *Mkk3*^{-/-} mice. (E) Heart weight to tibia length ratio in WT (*n*=13) and *Mkk3*^{-/-} (*n*=7) mice at 9 weeks of age. Data in E are mean ± SEM. (*n*=7–13). ***P*<0.01 (Unpaired *t*-test).

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698

699 **Figure 6. Loss of p38γ/δ in cardiomyocytes rescues the cardiac hypertrophy**

700 **induced by MKK6 deficiency.** All phenotypes shown come from 9-week-old mice.

701 (A) Heart weight to tibia length ratio in WT (*n*=5), *Mkk6*^{-/-} (*n*=10), *p38*^{γ-/-} (*n*=7) and

702 *Mkk6*^{-/-} *p38*^{γ-/-} (*n*=7) mice. 1-way ANOVA followed by Tukey's post test. (B)

703 Representative H&E-stained transverse heart sections from WT, *p38*^{γ-/-} and *Mkk6*^{-/-}

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704 p38 $\gamma^{-/-}$ mice. Scale bars: 1 mm. (C) Representative FITC-WGA staining and
705 corresponding quantification of cardiomyocyte cross-sectional area in WT ($n=5$), p38 $\gamma^{-/-}$
706 ($n=4$) and *Mkk6* $^{-/-}$ p38 $\gamma^{-/-}$ ($n=5$) mice. Scale bars: 25 μ m. 1-way ANOVA followed by
707 Tukey's post test. (D) Heart weight to tibia length ratio in MCK-Cre ($n=8$), *Mkk6* $^{-/-}$
708 ($n=10$), p38 δ^{MCK-KO} ($n=8$), and *Mkk6* $^{-/-}$ p38 δ^{MCK-KO} ($n=8$) mice. Kruskal-Wallis test with
709 Dunn's post-test (## $P<0.01$ Mann-Whitney test). (E) Representative H&E-stained
710 transverse heart sections from MCK-Cre, p38 δ^{MCK-KO} , and *Mkk6* $^{-/-}$ p38 δ^{MCK-KO} mice.
711 Scale bars: 1 mm. (F) Representative FITC-WGA staining and corresponding
712 quantification of cardiomyocyte cross-sectional area in MCK-Cre ($n=5$), p38 δ^{MCK-KO}
713 ($n=5$), and *Mkk6* $^{-/-}$ p38 δ^{MCK-KO} ($n=5$) mice. 1-way ANOVA followed by Tukey's post
714 test. Scale bars: 25 μ m. The same data from *Mkk6* $^{-/-}$ mice was used in (A) and (D).
715 Means $\pm$ SEM are shown. * $P<0.05$; ** $P<0.01$; *** $P<0.001$; ## $P<0.01$.

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Figure 7. Hyperactivation of mTOR signaling drives cardiac hypertrophy in *Mkk6*

***-/-* mice. (A, B) Immunoblot analysis of mTOR signaling pathway activity (A) and**

activation status of translation factors (B) in heart lysates from 9-week-old WT and *Mkk6*^{-/-} mice. (C) *In vivo* measurement of protein synthesis. Mice were injected intraperitoneally with 0.040 $\mu\text{mol g}^{-1}$ puromycin dissolved in 100 μl PBS. Exactly 30 min after injection, tissues were extracted and frozen in liquid N₂ for immunoblot analysis with anti-puromycin antibody. (D) Protein content of WT (*n*=8) and *Mkk6*^{-/-} (*n*=5) hearts measured as the protein-DNA ratio. Mann-Whitney test. (E) Heart weight to tibia length ratio in WT (*n*=5-7) and *Mkk6*^{-/-} (*n*=6-7) mice after rapamycin treatment. Mice received daily intraperitoneal injections with rapamycin (2 mg kg⁻¹ per day) or vehicle from weeks 4 to 9 after birth. 2-way ANOVA followed by Tukey's posttest. (F) Representative H&E-stained transverse heart sections after treatment. Scale bars: 1 mm. (G) Representative FITC-WGA staining and corresponding quantification of cardiomyocyte cross-sectional area from WT (*n*=4) and *Mkk6*^{-/-} (*n*=4) mice hearts after rapamycin treatment. 2-way ANOVA followed by Tukey's post test. Scale bars: 25 μm . Data in D, E and G are mean $\pm$ SEM. **P*<0.05; ***P*<0.01; ****P*<0.001.

733

734 **Figure 8. Model for p38 γ/δ activation mediated cardiac hypertrophic growth.** In a
735 physiological context, MKK3-p38 γ/δ pathway promotes normal cardiac growth through
736 the activation of mTOR signaling pathway. MKK6 deficiency stimulates the
737 hyperactivation of MKK3-p38 γ/δ and the consequent increase in mTOR activity, which
738 drives increased cardiac hypertrophy.