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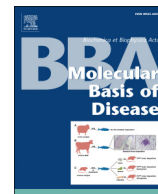
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## Highlights

**Met signaling in cardiomyocytes is required for normal cardiac function in adult mice***Biochimica et Biophysica Acta xxx (2013) xxx – xxx*

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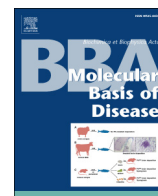
- *In vivo* genetic inactivation of Met in cardiomyocytes leads to heart dysfunction.
- Met signaling prevents reactive oxygen species accumulation in cardiomyocytes.
- HGF/Met acting through p38 $\alpha$  MAPK up-regulates antioxidant enzymes in cardiomyocytes.

Q5 Supplementary material



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## Met signaling in cardiomyocytes is required for normal cardiac function in adult mice

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### ABSTRACT

Hepatocyte growth factor (HGF) and its receptor, Met, are key determinants of distinct developmental processes. Although HGF exerts cardio-protective effects in a number of cardiac pathologies, it remains unknown whether HGF/Met signaling is essential for myocardial development and/or physiological function in adulthood. We therefore investigated the requirement of HGF/Met signaling in cardiomyocyte for embryonic and postnatal heart development and function by conditional inactivation of the Met receptor in cardiomyocytes using the Cre- $\alpha$ -MHC mouse line (referred to as  $\alpha$ -MHC*Met*-KO). Although  $\alpha$ -MHC*Met*-KO mice showed normal heart development and were viable and fertile, by 6 months of age, males developed cardiomyocyte hypertrophy, associated with interstitial fibrosis. A significant upregulation in markers of myocardial damage, such as  $\beta$ -MHC and ANF, was also observed. By the age of 9 months,  $\alpha$ -MHC*Met*-KO males displayed systolic cardiac dysfunction. Mechanistically, we provide evidence of a severe imbalance in the antioxidant defenses in  $\alpha$ -MHC*Met*-KO hearts involving a reduced expression and activity of catalase and superoxide dismutase, with consequent reactive oxygen species accumulation. Similar anomalies were observed in females, although with a slower kinetics. We also found that Met signaling down-regulation leads to an increase in TGF- $\beta$  production and a decrease in p38MAPK activation, which may contribute to phenotypic alterations displayed in  $\alpha$ -MHC*Met*-KO mice. Consistently, we show that HGF acts through p38 $\alpha$  to upregulate antioxidant enzymes in cardiomyocytes. Our results highlight that HGF/Met signaling in cardiomyocytes plays a physiological cardio-protective role in adult mice by acting as an endogenous regulator of heart function through oxidative stress control.

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### 1. Introduction

The hepatocyte growth factor (HGF) receptor, Met, is a tyrosine kinase receptor expressed in a wide variety of cell types. It regulates

several cellular functions, including proliferation, scattering, differentiation and survival, either during development or under some pathological conditions [1–3]. Upon HGF binding, Met is autophosphorylated in different tyrosine residues, leading to the recruitment of several SH2-domain containing proteins through the multifunctional docking site located in its carboxy-terminal tail [4]. Both HGF and Met are essential during development as shown by defects in placenta, liver, muscle, and nerves [5–10]. The embryonic lethality of *met* mutants imposes the use of conditional inactivation strategies to explore the HGF/Met functional requirement during embryogenesis and adulthood.

Several studies have been performed to uncover the HGF/Met properties in the heart. It has been shown that HGF protects rats from myocardial infarction [11] and/or ischemia/reperfusion injury [12]. Post-

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infarction treatment with HGF also improves left ventricular remodeling and heart function in mice [13]. In addition, HGF improves heart functionality and promotes proliferation of myocardial progenitor cells in doxorubicin-induced cardiomyopathy [14]. It also prevents heart fibrosis, remodeling and dysfunction in a hamster model of cardiomyopathy [15]. In other models of cardiac damage, HGF also inhibits fibrosis and additionally stimulates angiogenesis [16]. Together, these findings indicate that HGF/Met axis exerts a cardio-protective role under pathological conditions. *In vitro* studies have shown that HGF induces the expression of cardiac markers in mesenchymal stem cells [17] and increases cardiac differentiation of embryonic stem cells [18]. In addition, stem cells express HGF, which improves its survival and that of cardiomyocytes and favors angiogenesis [16]. Both HGF and Met are expressed in cardiac myocytes during early cardiogenesis [19,20]. In a mouse model of HGF supra-physiological expression, it has been shown that neonatal cardiomyocytes express Met and respond to HGF by increasing proliferation [21]. However, it remains unknown whether endogenous activity levels of HGF/Met are required for heart development and/or physiology after birth.

In this study, we have genetically evaluated the role played by the HGF/Met axis in developing and adult hearts using a conditional knock-out mouse model lacking Met signaling in cardiomyocytes. In particular, we conditionally deleted Met in cardiomyocytes by combining met floxed mice [22] with a transgenic line expressing the Cre recombinase under the control of the  $\alpha$ -myosin heavy chain ( $\alpha$ -MHC) promoter [23]. The  $\alpha$ -MHC promoter is active in mouse embryos throughout the myocardium starting from E8 [24]. Previous reports have shown that by E12.5,  $\alpha$ -MHC is highly expressed in atria and to a lesser extent in the ventricles, whereas its expression increases in the ventricles after birth [25]. Therefore, the  $\alpha$ -MHC-Cre line allows *c-met* inactivation in cardiomyocytes at early heart developmental stages.

Using the cardiomyocyte conditional Met knockout, we have found that Met signaling in cardiomyocytes appears to be dispensable for heart development. However, it is required to protect cardiomyocytes from oxidative stress, thus preventing cardiomyocyte hypertrophy, heart fibrosis, and heart dysfunction in adult mice. Together, these studies unveil that HGF/Met signaling in cardiomyocytes is a key regulator for proper heart function.

## 2. Materials and methods

### 2.1. Generation of cardiomyocyte *c-met* knock-out mice and genotyping

The animals used in our research program were handled in compliance with the institutional and European Union guidelines for animal care and welfare. All mouse lines were maintained and bred at the UCM facility, allowed food and water *ad libitum* and routinely screened for pathogens in accordance with FELASA (Federation of European Laboratory Animal Science Associations) procedures.

*c-met* flox/flox (wt) mouse line (in a CD1 genetic background), which carries *c-met* alleles with exon 16 flanked by loxP sites, was obtained from Dr. S. Thorgeirsson [22]. This mouse was crossed with  $\alpha$ -MHC-Cre transgenic mouse in CD1 background (obtained from Dr. M. D. Schneider [23]) to generate  $\alpha$ -MHC-Cre/+; *c-met* flox/+ mice, which were then crossed with *c-met* flox/flox mice. Cre-mediated excision led to a null allele for *c-met*, which encodes an inactive Met receptor only in cardiomyocytes. Mice genotyping was performed by PCR analysis (Supplemental methods).

### 2.2. RT-qPCR analysis

After the isolation of total RNA, DNA and proteins using AllPrep DNA/RNA/Protein Mini Kit, RNA was reverse transcribed using SuperScript III RT kit (Qiagen, 18080) to generate cDNA. Then, real time PCR was performed using specific primers (see Supplemental methods). Quantification was performed calculating RQ ( $2^{-\Delta\Delta Ct}$ ), so referring the

values to control mice (Supplemental methods). An Invitrogen custom RNA profiling by RT-qPCR was performed for the analysis of anti- and pro-fibrotic genes and those involved in reactive oxygen species (ROS) metabolism (Supplemental methods).

### 2.3. Western-blot analysis

Western-blot analysis was carried out as previously described [26]. Proteins were separated by electrophoresis using Anderson or SDS-PAGE gels, and transferred to nitrocellulose membranes that were probed with the following antibodies: catalase (Sigma, C0979), superoxide dismutase-2 (SOD-2) (Upstate Biotech, 06-984),  $\beta$ -actin (Sigma A5441), c-Met (Santa Cruz Biotechnology sc-260), cytochrome c (BD 556433), porin (Sigma V2139), phospho-p38MAPK (Cell Signaling, 9211), and p38 $\alpha$  (Santa Cruz Biotechnology sc-535).

### 2.4. Histology

Hearts were processed for histology using conventional techniques involving paraformaldehyde fixation and paraffin embedding. The degree of interstitial fibrosis was assessed by image analysis on Mallory's trichrome stained tissue sections (see Supplemental methods). Laminin immunostaining of heart sections was done to delineate the cardiomyocyte profiles, measuring their areas with the ImageJ analysis package. Apoptotic cardiomyocytes were detected by using anti-desmin (Sigma, D1033) and anti-active caspase-3 antibodies (Promega, G74819).

### 2.5. ROS detection and quantification

Reactive oxygen species (ROS) accumulation in hearts was detected by staining of frozen sections with 1  $\mu$ M 2-hydroethidine (in PBS). The sections were then washed with PBS, mounted and analyzed by fluorescence microscopy. The number of stained nuclei was counted on digitalized images using ImageJ software. Alternatively, in hearts from 9-month-old mice, lipid peroxidation was determined by immunolocalization of 4-OH-Nonenal (Alpha Diagnostics, HNE11-S) in paraffin sections. Stained areas were assessed on digitalized images using ImageJ software.

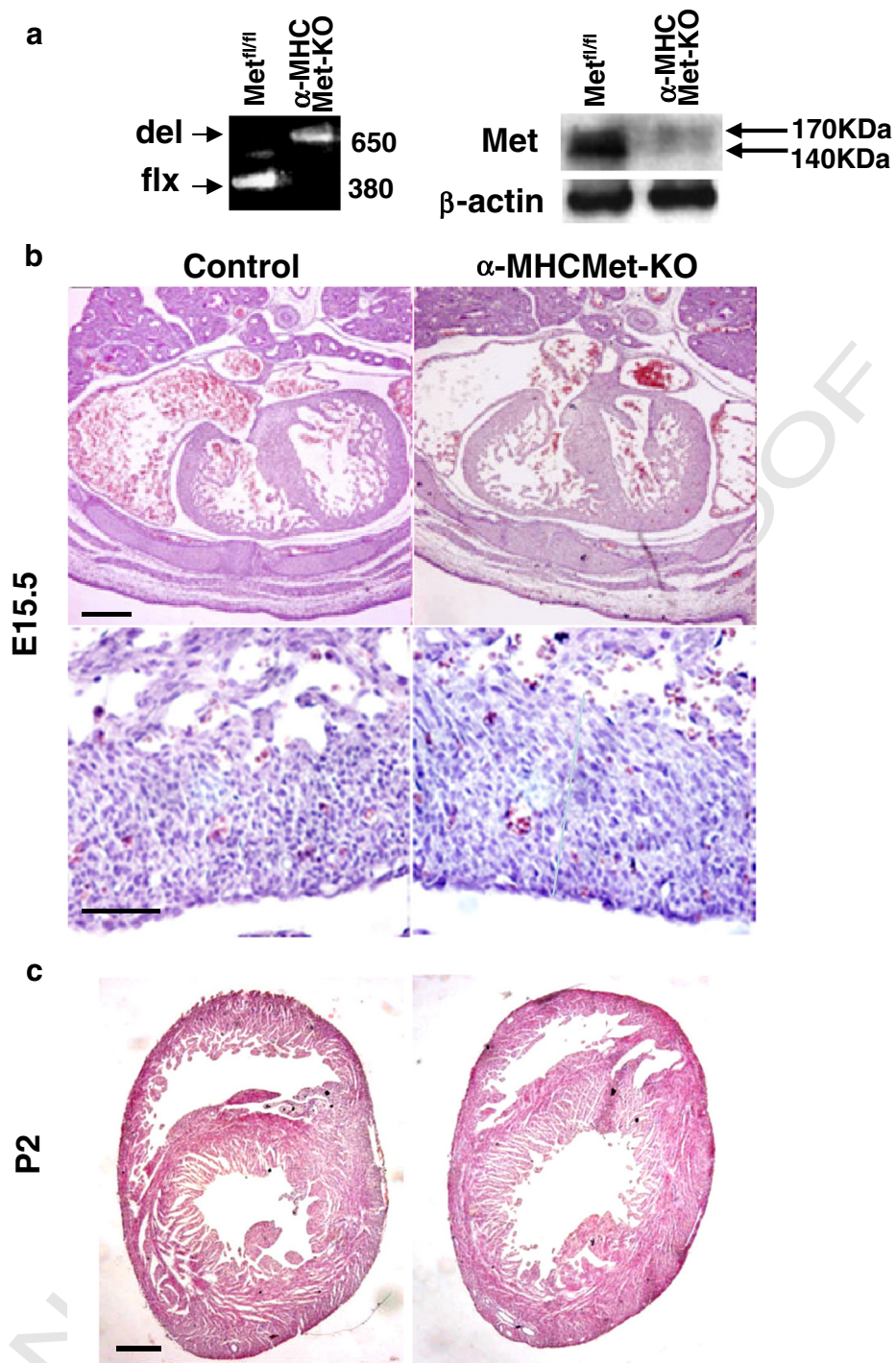
Carbonylated proteins were quantified as an indirect measurement of ROS [27]. Heart extracts were treated with 2,4-dinitrophenylhydrazine or maintained untreated (blank). Then, proteins were precipitated with TCA and absorbance at 360 nm was determined. The concentration of carbonylated proteins was calculated upon correction with the blanks and referred to protein content (Supplemental methods).

### 2.6. Measurement of catalase, SOD, and cytochrome c oxidase activities

Catalase activity was measured by quantification of peroxide decomposition spectrophotometrically at 240 nm. Superoxide dismutase (SOD) activity was quantified using a Kit (BioVision) (Supplemental methods). Cytochrome c oxidase activity was determined by quantification of cytochrome c oxidation spectrophotometrically at 550 nm.

### 2.7. Electrocardiographic measurements in conscious mice using radiotelemetry

Telemetric electrocardiogram (ECG) recordings in conscious mice were carried out using an implantable telemetry system with ECG transmitters (ETA-F10) (Data Sciences International, St. Paul, MN, USA). Recordings were taken every 3 h, 5 min/each, over a 24 h period and analyzed using ECG-auto software supplied by Data Systems (see extensive description in Supplemental methods).



**Fig. 1.** Conditional deletion of c-met in cardiomyocytes allows normal development and function of the heart in mice. a: Left panel: Representative PCR analysis showing a deletion (del) specific fragment (650 bp) from the Cre-recombined allele detected in E15.5 embryonic hearts from  $\alpha$ -MHCMet-KO mice after c-met exon 16 deletion and the floxed (flx) fragment (380 bp) present in met flox/flox hearts without Cre recombination (named as Met<sup>fl/fl</sup> or control) ( $n = 5$ ). Right panel: Western blot analysis of Met protein levels in hearts from E15.5 embryos. b and c: Heart sections from wt and  $\alpha$ -MHCMet-KO E15.5 embryos (b) and P2 newborn mice (c) stained with hematoxylin/eosin (scale bars: 250  $\mu$ m for b top panel and c, and 50  $\mu$ m for b low panel) ( $n = 10$ ).

## 185 2.8. Echocardiographic assessment of cardiac function and structure

186 Transthoracic echocardiography was performed on lightly anesthe-  
 187 tized mice using a Vivid 7 (GE Medical Systems) cardiac ultrasound  
 188 machine equipped with a 10- to 14-MHz transducer i13L (GE Medical  
 189 Systems), as previously described [28]. Measurements of chamber  
 190 dimensions and cardiac function were made offline (EchoPac Software,  
 191 GE Medical Systems). Structural and functional heart parameters were

calculated as previously described [28] (see extensive description in  
 Supplemental methods).

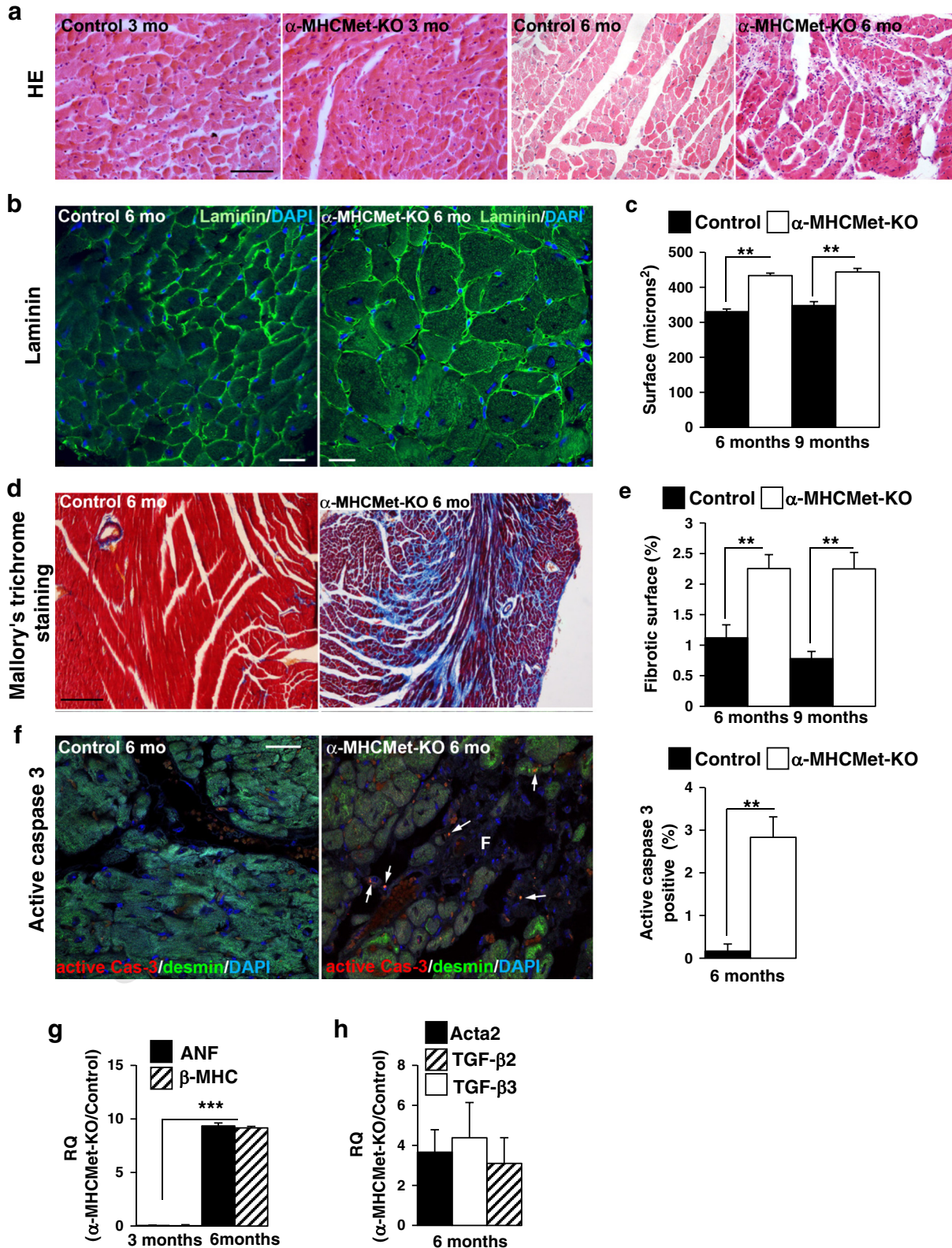
## 2.9. Assays in cardiomyocytes derived cell lines

Cardiomyocyte cell lines (wt and p38 $\alpha$   $-/-$ ) were generated from  
 E9.5 immortal mouse embryos expressing a temperature-sensitive large  
 T antigen (LTag) [29]. Before the experiments, cells were transferred to

198 37 °C in the absence of IFN- $\gamma$  to abolish LTA $\gamma$  expression. Neonatal  
 199 cardiomyocyte cell lines were obtained by immortalization of neonatal  
 200 mice (P4) cardiomyocytes by transfection with LTA $\gamma$ . For the experi-  
 201 ments, all the cell lines were maintained in a low serum medium  
 202 (2%) and treated with the Met inhibitor SU11274 (5  $\mu$ M) or TGF- $\beta$   
 203 (1 ng/mL) in the presence or absence of HGF (30 ng/mL, 16 h pretreat-  
 204 ment) or N-acetyl cysteine (2.5 mM) for 1–4 days as indicated.

## 2.10. Statistical analysis

205  
 206 Data are represented as means  $\pm$  SEM. The comparisons were made  
 207 between two experimental groups. An unpaired Student's *t*-test was  
 208 used, and alternatively, ANOVA test was carried out for comparisons  
 209 of more than two experimental groups. Differences between groups  
 210 were considered significant when  $p < 0.05$ .



### 3. Results

#### 3.1. Conditional inactivation of *Met* in cardiomyocytes does not overtly affect embryonic and postnatal heart development, but leads to cardiac anomalies in adult mice

In order to study the role played *in vivo* by *Met* in cardiomyocytes and to bypass embryonic lethality, we generated a mouse model with a selective inactivation of *Met* signaling in cardiomyocytes. To do it, we intercrossed the *c-met* floxed mouse, which carries *c-met* alleles with exon 16 flanked by loxP sites (*c-met<sup>fl/fl</sup>*) [22] with a transgenic mouse bearing the Cre recombinase under the  $\alpha$ -MHC promoter [23]. The resulting  $\alpha$ -MHC*Cre:met<sup>fl/fl</sup>* conditional knockout will be herein referred to as  $\alpha$ -MHC*Met-KO*. Because some strains of transgenic  $\alpha$ -MHC*Cre* mice expressing very high levels of Cre-recombinase in cardiomyocytes have been described to present heart anomalies [30], we checked the hearts from our  $\alpha$ -MHC*Cre* mice. They were similar to *c-met<sup>fl/fl</sup>* (considered as controls) in all experiments performed (Supplementary Fig. 1). So, both were indistinctly used as controls.

Efficiency of the *c-met* deletion was determined by PCR and Western-blot analysis. A deletion-specific fragment (650 bp) was amplified by PCR using DNA from E15.5  $\alpha$ -MHC*Met-KO* embryonic hearts. Consistently, a sharp decrease in c-Met protein levels was also found (Fig. 1a), confirming the efficient deletion of the *Met* floxed exon 16, as previously shown in other tissue-specific Cre lines [22,31,32]. The *Met* deletion was further confirmed in adult hearts (data not shown). Quantification revealed a *Met* deletion of around 70% and 90% in embryonic (E15.5) and adult  $\alpha$ -MHC*Met-KO* hearts, respectively (data not shown). The remaining *Met* expression corresponds most likely to that present in non-cardiomyocyte cells. No significant morphological differences were found between control and  $\alpha$ -MHC*Met-KO* mutant hearts, either during embryonic development or after birth (Fig. 1b and c). These data indicate that *Met* signaling in cardiomyocytes is dispensable for heart development.

Conditional  $\alpha$ -MHC*Met-KO* mutants were born following a Mendelian ratio. They displayed a normal lifespan and weight curve (not shown), thus indicating that other tissues had been preserved from *Met* inactivation. Therefore, the  $\alpha$ -MHC*Met-KO* mutant mice represent a unique genetic setting to investigate *Met* signaling requirement for heart function. This issue was assessed by performing a series of morphological, molecular, and functional studies on 3-, 6-, and 9-month-old  $\alpha$ -MHC*Met-KO* mice. It is well known that the gender may influence the molecular process underlying heart function, as shown by a number of studies in mice [33–35]. We therefore first focused our analyses on  $\alpha$ -MHC*Met-KO* males. Whereas morphological analysis of the heart did not reveal significant abnormalities in 3-month-old  $\alpha$ -MHC*Met-KO* males, we observed structural abnormalities in 6-month-old  $\alpha$ -MHC*Met-KO* mutant hearts (Fig. 2a, b, d). In particular, a significant increase in the cross-sectional area of cardiomyocytes was observed in  $\alpha$ -MHC*Met-KO* mutant hearts from 6- to 9-month-old males compared to controls (Fig. 2b, c and Supplementary Fig. 2a). In addition, myocardial fibrosis, diffused in some places and more prominent in defined areas, with cardiomyocyte disorganization and strong collagen deposition, was also found in the heart of 6- and 9-month-

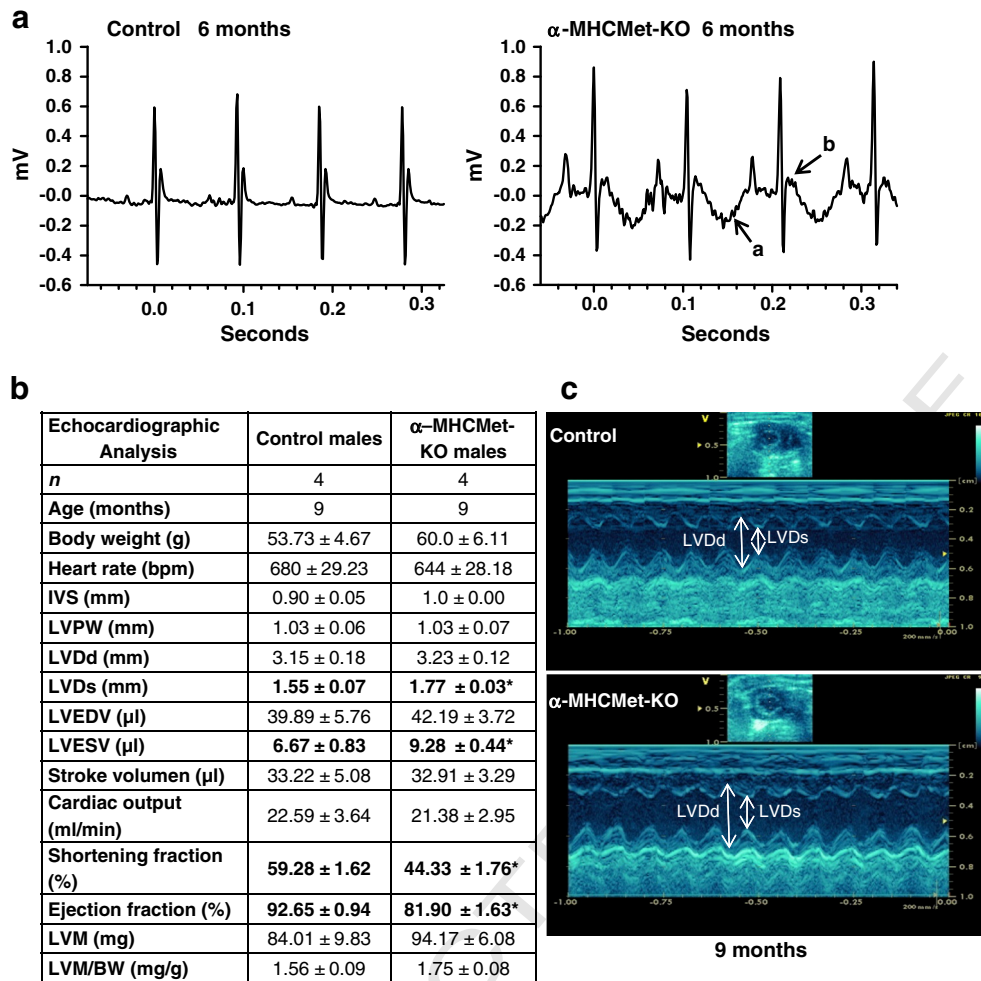
old  $\alpha$ -MHC*Met-KO* males as compared to controls (Fig. 2d, e and Supplementary Fig. 2b and c). As fibrosis can be a physiological response to cardiomyocyte death, we analyzed the presence of active caspase-3 in heart sections. We found an increase in the percentage of active caspase 3 positive cells, mainly on highly fibrotic areas of hearts from  $\alpha$ -MHC*Met-KO* males as compared to controls (Fig. 2f).

We next asked whether these morphological defects observed in  $\alpha$ -MHC*Met-KO* males were parallel to heart dysfunction markers. In particular, we performed RT-qPCR analyses to assess the levels of  $\beta$ -MHC and atrial natriuretic factor (ANF) mRNAs, known to be induced during hypertrophy [36,37]. No differences were observed in 3-month-old mutants (Fig. 2g). In contrast, we found increased mRNA expression levels of ANF, and  $\beta$ -MHC in hearts of 6-month-old  $\alpha$ -MHC*Met-KO* males as compared to controls (Fig. 2g and Supplementary Fig. 1a). Moreover, RT-qPCR expression analyses of pro-fibrotic genes such as TGF- $\beta$ 2 and TGF- $\beta$ 3, or fibrotic markers such as  $\alpha$ -smooth muscle actin (*Acta-2*) revealed significantly increased levels in  $\alpha$ -MHC*Met-KO* males as compared to controls (Fig. 2h).

We next assessed whether these morphological and molecular alterations impacted the heart electrical function by performing electrocardiography (ECG) analyses. Typical non-sedated mice ECG shows a J wave just after QRS complex, an almost isoelectric S-P interval and a P wave [38]. This typical ECG was observed in control mice (Fig. 3a). However, we found specific alterations in 6-month-old  $\alpha$ -MHC*Met-KO* males. Anomalies consisted in the reduction or disappearance of the J wave [38] and a negative deflection of the S-P interval ( $-0.21 \pm 0.02$  mV, versus  $-0.09 \pm 0.02$  mV in control mice,  $p < 0.01$ ), in contrast with the isoelectric S-P interval observed in control animals [38] (Fig. 3a). Although visual inspection suggested also an increased P wave in the  $\alpha$ -MHC*Met-KO* compared with control male mice, a detailed measurement of the wave amplitude ( $0.14 \pm 0.02$  mV in  $\alpha$ -MHC*Met-KO* and  $0.12 \pm 0.02$  mV in control mice ( $p > 0.1$ )) showed no statistically significant differences (Fig. 3a). However, data from echocardiographic analyses demonstrated no significant differences in cardiac output or left ventricular mass in  $\alpha$ -MHC*Met-KO* males (not shown). In contrast, echocardiographic analyses of 9-month-old  $\alpha$ -MHC*Met-KO* males revealed signs of systolic dysfunction characterized by a significantly higher left ventricular systolic diameter and volume compared to controls. Shortening fraction and ejection fraction were also significantly decreased (Fig. 3b and c). The LV posterior wall thickness/LV end systolic dimension ratio was slightly lower in 9-month-old  $\alpha$ -MHC*Met-KO* males ( $0.56 \pm 0.03$ ) than in controls ( $0.66 \pm 0.03$ ,  $p < 0.05$ ), which suggests the existence of a left ventricular dilation.

Morphological, molecular, and functional studies were also performed in females. Increased cardiomyocyte size and extensive areas of fibrosis were detected in 9-month-old  $\alpha$ -MHC*Met-KO* females (Supplementary Fig. 3a–c). Echocardiographic analysis revealed no significant differences between  $\alpha$ -MHC*Met-KO* and controls, although a similar tendency as that found in mutant males was observed (data not shown). Together, these studies showed that HGF/*Met* signaling in cardiomyocytes is dispensable during development and in young mice, whereas it is required for proper heart function in adult mice. Moreover, they indicate that males are more susceptible to an altered HGF/*Met* signaling than females.

**Fig. 2.** *Met* inactivation in cardiomyocytes leads to cardiomyocyte hypertrophy, fibrosis and apoptosis in adult mice. a: Heart sections from control and  $\alpha$ -MHC*Met-KO* males (3 and 6 months old) stained with hematoxylin/eosin (scale bar: 100  $\mu$ m) ( $n = 5$ ). b: Representative laminin immunostaining of heart sections from 6-month (mo) old males (control and  $\alpha$ -MHC*Met-KO*). c: Histogram showing the mean values  $\pm$  SEM of the transverse cardiomyocyte area in 6- and 9-month-old male hearts. Differences between control and  $\alpha$ -MHC*Met-KO* are significant (\*\* $p < 0.01$ ,  $n = 5$ ). d: Mallory's trichrome staining of collagen. Fibrotic areas (in blue) are present in 6-month-old  $\alpha$ -MHC*Met-KO* male hearts (scale bar: 200  $\mu$ m). e: Histogram showing the mean values  $\pm$  SEM of the fibrotic cardiac surface in 6- and 9-month-old male hearts (control and  $\alpha$ -MHC*Met-KO*). \*\* $p < 0.01$ , control as compared with  $\alpha$ -MHC*Met-KO* ( $n = 5$ ). f: Immunolocalization of active caspase-3 in 6-month-old male hearts (left) and histogram showing the mean values  $\pm$  SEM of the percentage of active caspase 3 positive cells (right) ( $n = 4$ ). Positive cells (arrows) can be observed in the fibrotic areas of  $\alpha$ -MHC*Met-KO* hearts (scale bar: 25  $\mu$ m). Some of them show remaining desmin immunoreactivity (in green), suggesting they are apoptotic cardiomyocytes. Note the depletion of desmin in cardiomyocytes from the fibrotic areas. g: Quantification of ANF and  $\beta$ -MHC mRNAs expression by RT-qPCR in hearts from 3- and 6-month-old males, comparing  $\alpha$ -MHC*Met-KO* with controls of the same age. RQ values  $\pm$  SEM are shown in the histogram ( $n = 5$ ). A significant increase (\*\* $p < 0.001$ ) is produced in 6-month-old MHC*Met-KO* males. h: Significant increased (\* $p < 0.05$ ) expression of TGF- $\beta$ 2, TGF- $\beta$ 3 and *Acta2* mRNAs in 6-month-old mutant males as compared with controls detected by RT-qPCR analysis (mean RQ values  $\pm$  SEM are shown,  $n = 3$ ).



**Fig. 3.** Conditional deletion of *c-met* in cardiomyocytes leads to heart dysfunction in 9-month-old mice. **a:** Representative electrocardiogram (ECG) of an  $\alpha$ -MHCMet-KO compared with a control male (6 months old). The figure shows a negative deflection in the S-P segment (**a**) and a lower J wave (**b**). **b:** Echocardiography analysis in 9-month-old males. The following parameters are shown: IVS, interventricular septum thickness; LVPW, left-ventricular posterior wall thickness; LVDD, left-ventricular diastolic dimension; LVDs, left-ventricular systolic dimension; LVDEV, left-ventricular end-diastolic volume; LVESV, left-ventricular end-systolic volume; LVM, left ventricular mass. All data are expressed as mean  $\pm$  SEM. \**p* < 0.05, control as compared with  $\alpha$ -MHCMet-KO. **c:** Representative echocardiographic images of an  $\alpha$ -MHCMet-KO compared with a control male.

### 3.2. Loss-of-function of *Met* signaling disrupts the antioxidant defenses in cardiomyocytes, leading to an increase in ROS accumulation and altered mitochondria in adult mice heart

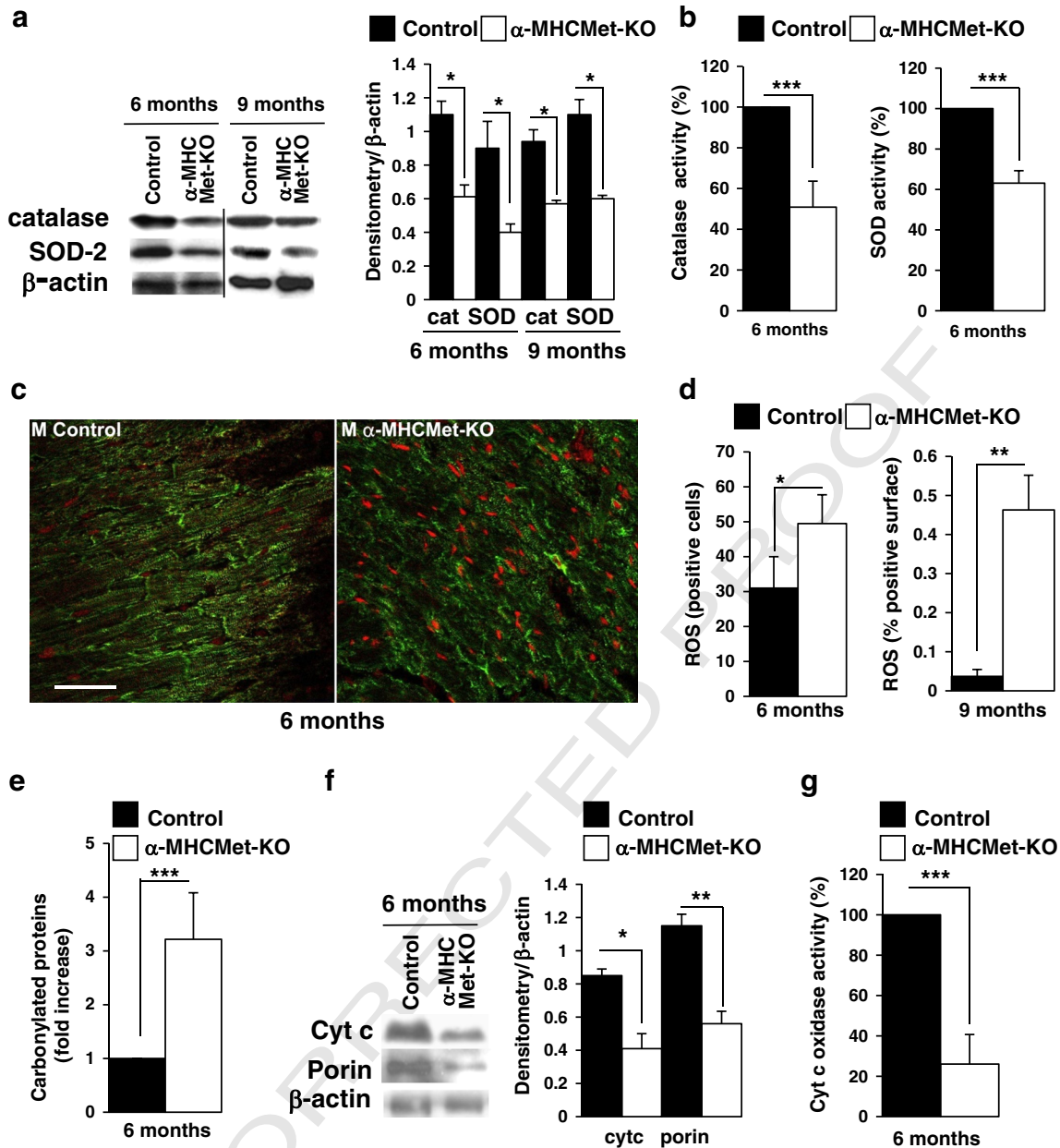
The oxidative stress increases during aging, leading to cardiac alterations, including hypertrophy and heart dysfunction [39]. As HGF/*Met* signaling regulates the antioxidant defenses in different cells types by preventing ROS accumulation [40–42], we investigated whether heart defects observed in adult  $\alpha$ -MHCMet-KO males were linked to an imbalance between pro- and anti-oxidant mechanisms. We found that protein levels of the antioxidant enzymes, catalase and SOD-2, were reduced in the heart of 6- and 9-month-old  $\alpha$ -MHCMet-KO males as compared to controls (Fig. 4a and Supplementary Fig. 1b). Consistently, catalase and SOD-2 activities were also decreased in  $\alpha$ -MHCMet-KO males (Fig. 4b). We also found that reactive oxygen species were accumulated in cardiomyocytes of  $\alpha$ -MHCMet-KO males as shown by 2-hydroethidine staining and 4-OH-nonenal immunolocalization in tissue sections from 6- to 9-month-old mice, respectively (Fig. 4c and d). In addition, carbonylated protein levels increased in 6-month-old mutant males (Fig. 4e), as a readout of proteins oxidation by ROS.

Because ROS accumulation can either be a consequence of an altered mitochondrial function and/or lead to mitochondrial damage, we next investigated whether  $\alpha$ -MHCMet-KO 6-month-old male hearts presented alterations in mitochondria. We found that the mitochondrial proteins,

porin and cytochrome *c* were significantly decreased in mutants as compared to controls (Fig. 4f). Consistently, cytochrome *c* oxidase activity was also reduced in  $\alpha$ -MHCMet-KO hearts (Fig. 4g). In contrast, we did not observe any alteration in anti-oxidant enzymes, ROS accumulation, carbonylated proteins or mitochondrial proteins in 6-month-old  $\alpha$ -MHCMet-KO females, consistent with normal morphology and function, as previously assessed (Supplementary Fig. 3). However, in 9-month-old mutant female hearts anti-oxidant enzymes levels significantly decreased and ROS accumulation was detected (Supplementary Fig. 4c), according to the presence of fibrosis and the increased cardiomyocytes size (Supplementary Fig. 3). Altogether, these findings strongly indicate that HGF/*Met* signalling is required for normal mitochondrial function and redox homeostasis in adult cardiomyocytes.

### 3.3. *Met* acts on p38 MAPK to increase antioxidant enzymes levels and counteracts TGF- $\beta$ effect in cardiomyocytes

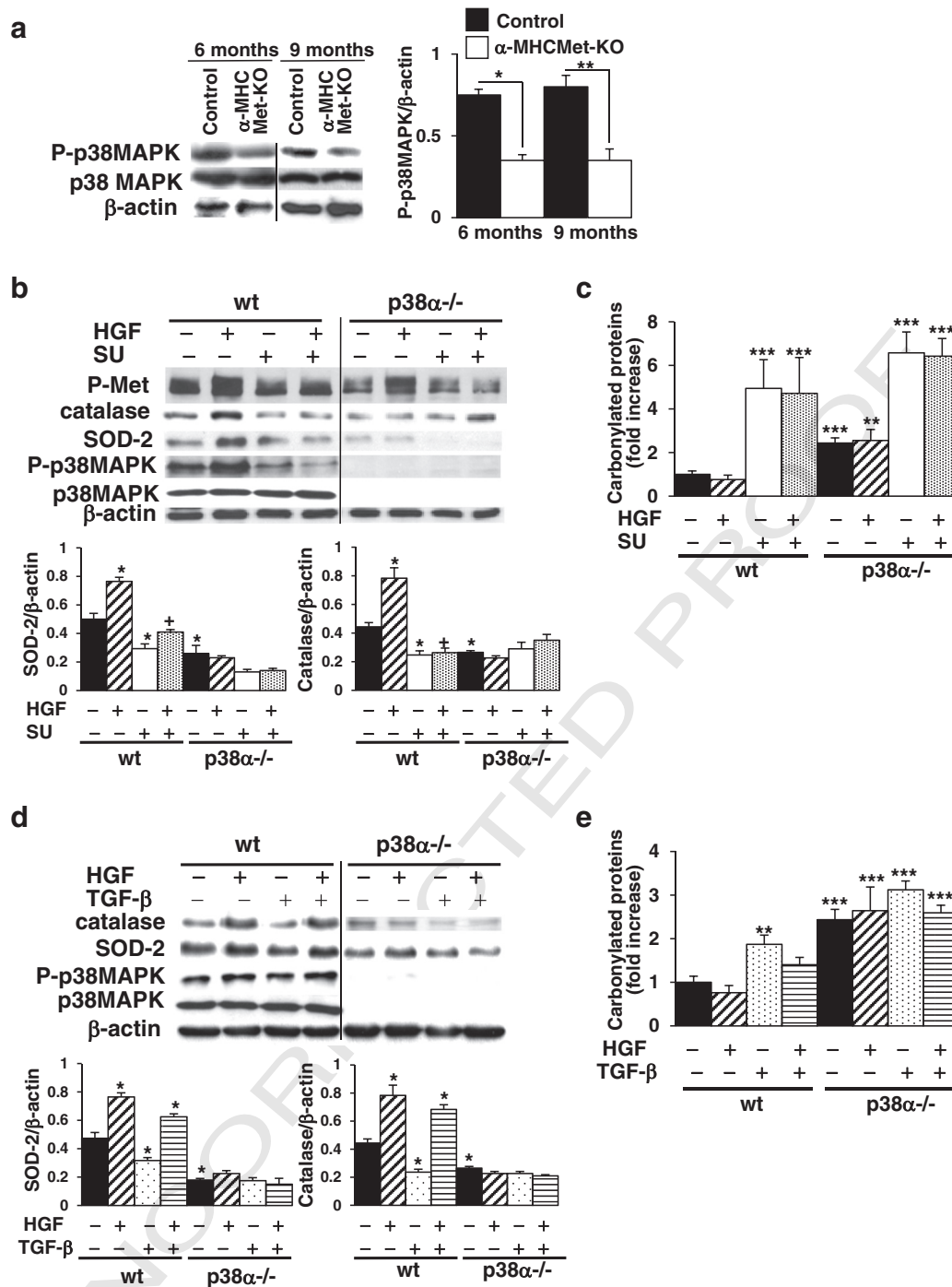
We next aimed at uncovering the molecular mechanism by which HGF/*Met* signaling in cardiomyocytes ensures proper levels of antioxidant enzymes. As it has been shown that p38 $\alpha$  MAPK regulates the expression of SOD-2 and catalase in mouse embryonic fibroblasts (MEFs) [43], we determined P-p38MAPK levels, as a readout of p38 activity in control and  $\alpha$ -MHCMet-KO hearts. Notably, we found that P-p38MAPK levels were significantly decreased in 6- and 9-month-old mice  $\alpha$ -MHCMet-KO



**Fig. 4.** Changes in anti-oxidant enzymes, ROS levels and mitochondrial proteins in adult  $\alpha$ -MHCMet-KO male hearts. **a:** Representative Western-blot analysis of catalase and superoxide dismutase-2 (SOD-2) protein levels in  $\alpha$ -MHCMet-KO and control hearts from 6- to 9-month-old males normalized with  $\beta$ -actin (left). The histogram shows the mean values  $\pm$  SEM of the densitometry of the blots from different experiments ( $n = 4$ ): catalase and SOD-2 versus  $\beta$ -actin.  $*p < 0.05$ , control as compared with mutants (right). **b:** Catalase and SOD-2 activities from 6-month-old males is shown as the percentage of the activities of control hearts (mean values  $\pm$  SEM),  $***p < 0.001$ , control as compared with  $\alpha$ -MHCMet-KO ( $n = 4$ ). **c:** Representative sections showing accumulation of ROS (detected by 2-hydroethidine staining) in cardiomyocytes from 6-month-old mutant males. **d:** Histograms show the mean values  $\pm$  SEM ( $n = 3$ ) of the number of 2-hydroethidine stained nuclei by microscopy field in cardiac tissue sections from 6-month-old males (left) and the percentage of 4-OH-nonenal immunoreactive surface in hearts from 9-month-old males (right).  $*p < 0.05$  and  $**p < 0.01$ , control as compared with  $\alpha$ -MHCMet-KO. **e:** Histograms represent the fold increase of carbonylated protein levels (mean values  $\pm$  SEM,  $n = 4$ ) present in heart extracts from  $\alpha$ -MHCMet-KO males referred to controls (6 months old).  $***p < 0.001$ ,  $\alpha$ -MHCMet-KO as compared with control mice. **f:** Representative cytochrome c (Cyt c) and porin Western-blot (left) and densitometry of blots (porin and cyt c versus  $\beta$ -actin) (right) from 6-month-old male hearts ( $n = 4$ ).  $*p < 0.05$  and  $**p < 0.01$ , control as compared with  $\alpha$ -MHCMet-KO. **g:** Cyt c oxidase activity is significantly decreased ( $***p < 0.001$ ) in 6-month-old  $\alpha$ -MHCMet-KO as compared with control males. Histograms represent the percentage of the activities in the mutants as compared to controls (mean values  $\pm$  SEM,  $n = 4$ ).

362 hearts (Fig. 5a and Supplementary Fig. 4g). We therefore studied whether  
 363 p38 $\alpha$  was required for HGF/Met-mediated regulation of antioxidant en-  
 364 zymes in cardiomyocytes through the use of pharmacological approaches  
 365 in genetically modified embryonic cardiomyocyte cell lines generated  
 366 from wt or p38 $\alpha$  deficient mice (p38 $\alpha$ <sup>-/-</sup>). We found decreased  
 367 catalase and SOD-2 protein levels in p38 $\alpha$ <sup>-/-</sup> as compared to wt  
 368 cardiomyocytes (Fig. 5b). Met inhibition with SU11274, detected by the  
 369 decrease in Met phosphorylation (Fig. 5b), reduced the levels of catalase,  
 370 SOD-2 and P-p38 MAPK in wt cells, but not in p38 $\alpha$ <sup>-/-</sup> cells (Fig. 5b).  
 371 These findings were further confirmed using neonatal cardiomyocytes

(data not shown). Moreover, HGF stimulation increased the levels of catalase,  
 372 SOD-2 and P-p38 MAPK proteins in wt, but not in p38 $\alpha$ <sup>-/-</sup> wt  
 373 cardiomyocytes (Fig. 5b). These findings indicate that HGF/Met acts  
 374 through p38 MAPK to increase catalase and SOD-2 levels. According to  
 375 this, an increase in the levels of carbonylated proteins was detected in  
 376 wt cardiomyocytes treated with SU11274 (Fig. 5c). HGF treatment  
 377 (alone) slightly decreased them. As expected, HGF had no effect when  
 378 used in combination with SU11274. Consistently, higher basal levels of  
 379 carbonylated proteins were found in p38 $\alpha$ <sup>-/-</sup> cardiomyocytes as compared  
 380 to those of wt cells, which were further increased upon SU11274  
 381



**Fig. 5.** HGF/Met signaling increases antioxidant enzymes in cardiomyocytes acting through p38 $\alpha$  MAPK. Effect on TGF- $\beta$  mediated down-regulation and ROS accumulation. a: Representative Western-blot analysis of P-p38MAPK and total p38 in hearts from 6- to 9-month-old males (left) and densitometric analyses of the blots (referred to  $\beta$ -actin,  $n = 4$ ). b: Upper panel, representative Western-blot analysis of catalase, SOD-2, P-Met, P-p38MAPK and total p38 protein levels in wt and p38 $\alpha$ <sup>-/-</sup> cardiomyocytes maintained untreated or treated with the Met inhibitor, SU11274 (SU) (3 days) and/or HGF (16 h pre-incubation), as indicated. Lower panel, histograms show the mean values  $\pm$  SEM ( $n = 3$ ) of the densitometric analyses of catalase and SOD-2 blots (referred to  $\beta$ -actin). \* $p < 0.05$ , as compared with wt control cells; + $p < 0.05$ , as compared with HGF treated wt cells. c: Levels of carbonylated proteins in wt and p38 $\alpha$ <sup>-/-</sup> cardiomyocytes treated as indicated in b section. The histogram shows the fold increase referred to control values from wt cells (mean values  $\pm$  SEM ( $n = 3$ )). \*\* $p < 0.01$  and \*\*\* $p < 0.001$ , as compared with wt control. d: Upper panel, representative Western-blot analysis of catalase, superoxide dismutase-2, P-p38MAPK and total p38 protein levels in wt and p38 $\alpha$ <sup>-/-</sup> cardiomyocytes maintained in the absence or presence of TGF- $\beta$  (3 days) and/or HGF (16 h pre-incubation), as indicated. Lower panel, histograms showing the mean values  $\pm$  SEM ( $n = 3$ ) of the densitometric analyses of catalase and SOD-2 blots (referred to  $\beta$ -actin). \* $p < 0.05$ , as compared with wt control. e: Levels of carbonylated proteins in wt and p38 $\alpha$ <sup>-/-</sup> cardiomyocytes treated as indicated in d section. The histogram shows the fold increase referred to control values from wt cells (mean values  $\pm$  SEM ( $n = 3$ )). \*\* $p < 0.01$  and \*\*\* $p < 0.001$ , as compared with wt control.

treatment. No changes were observed upon treatment with HGF (either alone or in combination with SU11274).

The increased levels of TGF- $\beta$  observed in  $\alpha$ -MHC Met-KO hearts (Fig. 2h) prompted us to investigate whether the levels of antioxidant enzymes were influenced by TGF- $\beta$ . We found that TGF- $\beta$  led to a

decrease in catalase and SOD-2 protein levels, mainly in wt cardiomyocytes (Fig. 5d). Intriguingly, HGF counteracted the decrease in catalase and SOD-2 protein levels mediated by TGF- $\beta$  in wt cardiomyocytes (Fig. 5d). Accordingly, HGF activated p38 $\alpha$ , both in the absence and presence of TGF- $\beta$  (Fig. 5d). In agreement with all these

392 changes in SOD-2 and catalase expression, TGF- $\beta$  increased the levels of  
 393 carbonylated proteins in wt cardiomyocytes (Fig. 5e). HGF slightly de-  
 394 creased them, but this effect was not statistically significant. In  
 395 p38 $\alpha$ <sup>-/-</sup> cardiomyocytes, TGF- $\beta$  did not further increase its intrinsic  
 396 high basal levels of carbonylated proteins and HGF had no effect on  
 397 them.

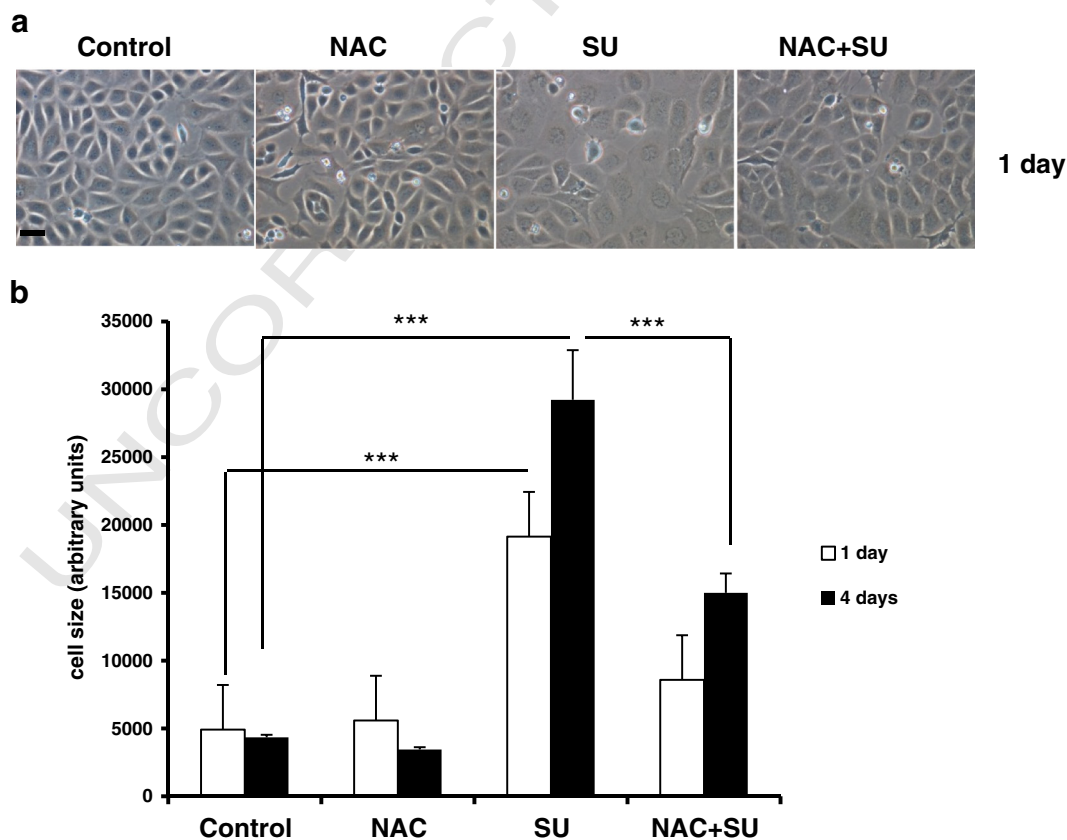
398 Taken together, these findings indicate that HGF/Met acts through  
 399 p38 $\alpha$  MAPK to increase the levels of antioxidant enzymes in cardio-  
 400 myocytes and to avoid ROS accumulation, thus partially counteracting  
 401 the effects elicited by TGF- $\beta$ .

#### 402 3.4. Increased cardiomyocyte size, occurring in the absence of Met signaling, 403 is prevented by antioxidant treatment

404 As alteration of Met signaling in cardiomyocytes leads to decreased  
 405 antioxidant enzymes levels, it is tempting to speculate that accumula-  
 406 tion of ROS in  $\alpha$ -MHCMet-KO hearts could be responsible for the in-  
 407 creased size of cardiomyocytes. This would be coherent with previous  
 408 studies showing that ROS accumulation is responsible for cardio-  
 409 myocyte hypertrophy as it was observed in cardiomyocytes isolated  
 410 from Sirt3-deficient mice [44]. We therefore explored this hypothesis  
 411 using wild-type neonatal cardiomyocyte cell lines. Remarkably, we  
 412 found that Met inhibition led to an increase in cell size in a time-  
 413 dependent manner (Fig. 6a, b). Treatment of cardiomyocytes with the  
 414 antioxidant N-acetyl-cysteine partially prevented the increase in the  
 415 size of cells exposed to the Met inhibitor, SU11274 (Fig. 6a, b). These  
 416 findings support that ROS accumulation might account for the hypertro-  
 417 phy of cardiomyocytes occurring in the absence of HGF/Met signaling.  
 418 Additionally, the fact that Met inhibition itself leads to an increase in  
 419 cardiomyocyte cell size suggests the existence of an autocrine signaling  
 420 loop.

#### 421 4. Discussion

422 Genetically modified mouse models have unveiled several genes in-  
 423 volved in heart development and in regulation of adult heart physiology.  
 424 However, key players for adult heart function and homeostasis remain to  
 425 be uncovered. Their identification would improve our understanding of  
 426 heart dysfunction, uncover new diagnostic markers and be instrumental  
 427 for therapy and/or regenerative interventions. HGF/Met is an attractive  
 428 signal as administration of exogenous HGF has a cardio-protective role  
 429 in models of heart damage [11–13]. By exploring *in vivo* the role played  
 430 by Met in heart during embryogenesis and in adulthood through selec-  
 431 tive inactivation of the *met* gene in cardiomyocytes, we demonstrated  
 432 that while it is not essential for heart development, Met is required for  
 433 heart homeostasis in adult mice. Intriguingly, Met becomes progressively  
 434 required during aging. Whereas 3-month-old  $\alpha$ -MHCMet-KO mutant  
 435 hearts are normal, hearts from 6-month-old males show an increase  
 436 in cardiomyocyte size and myocardial fibrosis. Molecular markers of  
 437 cardiac damage, such as ANF or  $\beta$ -MHC are up-regulated in  $\alpha$ -MHCMet-  
 438 KO males. This is accompanied by abnormal ECG, characterized by a  
 439 marked depression of the S-P segment, a feature reported in rats with  
 440 isoproterenol-induced cardiac hypertrophy [45]. As age increases,  
 441 lack of HGF/Met signaling impacts heart function, so 9-month-old  
 442  $\alpha$ -MHCMet-KO males suffer left ventricle systolic dysfunction. In addi-  
 443 tion, taking into account that, although the LV posterior wall thickness/  
 444 LV end systolic dimension ratio is slightly lower in 9-month-old  
 445  $\alpha$ -MHCMet-KO than in wt males, the ventricular mass/body weight  
 446 ratio is similar, which suggests the existence of heart dilation rather  
 447 than hypertrophy. Heart dysfunction of  $\alpha$ -MHCMet-KO mutants is  
 448 paralleled by a significant reduction in protein levels and enzymatic  
 449 activities of catalase and SOD-2 in the heart of  $\alpha$ -MHCMet-KO mutants,  
 450 accompanied by ROS accumulation. These alterations were found in



**Fig. 6.** Treatment with antioxidants partially prevents the increase in cardiomyocyte cell size induced by Met inhibition. Immortalized neonatal wt cardiomyocytes were maintained in the absence or presence of the Met inhibitor SU11274 (SU) and/or N-acetyl cysteine (NAC) for 1 or 4 days as indicated. a: Representative phase-contrast microscopy images after 1 day of treatment (scale bar: 25  $\mu$ m). b: Quantification of cardiomyocytes size after treatment, expressed as arbitrary units (\*\**p* < 0.001, *n* = 3).

both male and females. The delayed dysfunction observed in  $\alpha$ -MHC*Met-KO* females is most likely due to the existence of estrogen-induced cardio-protective mechanisms, which have been extensively studied [33–35] and which might partially compensate HGF/Met requirement for heart function in young females.

The cardio-protective role of Met during aging and in the absence of experimentally induced damage is in agreement with the HGF/Met properties reported in experimental models of human pathologies such as myocardial infarction [11,13], ischemia/reperfusion injury [12], or other models of cardiomyopathy, where fibrosis is produced [15]. However, in all these studies it was not possible to clarify in which cell types HGF/Met signaling was required, as HGF can activate Met in most of the cell types present in the heart. Thus, some of the HGF effects previously described could be mediated by Met activation in non-cardiomyocyte cell types, such as fibroblasts, coronary endothelium or vascular smooth muscle. It is also important to highlight that HGF is a potent pro-angiogenic factor and its effect promoting neovascularization would be cardioprotective *per se* [16,46]. The inhibition of Met signaling in myocardium, leaving intact the angiogenic roles of HGF on the coronary endothelium, allows excluding that the observed phenotype can be due to a vascular defect. Thus, our genetic mouse model determines *in vivo* a cell autonomous requirement of HGF/Met signaling in cardiomyocytes for their maintenance and function. Although our results clearly suggest a direct effect of HGF on mature cardiomyocytes, we cannot formally exclude that HGF signaling may also influence the expansion of cardiomyocyte progenitors as they express  $\alpha$ -MHC [47]. In fact, the improved heart functionality induced by HGF treatment in doxorubicin-induced cardiomyopathy [14] or the regeneration produced in an infarcted myocardium [48,49], could be explained by enhanced myocardial progenitor cells proliferation and/or migration. If the effect on cardiomyocyte progenitors is confirmed, HGF/Met signaling would be not only cardioprotective, but also involved in cardiac homeostasis.

The herein described property of HGF/Met signaling to protect cardiomyocytes from ROS-induced damage is in agreement with previous studies performed under non-physiological conditions. For example, HGF protects cultured adult rat cardiomyocytes from apoptosis induced by oxidative stress upon serum deprivation, treatment with daunorubicin, or hydrogen peroxide [50]. A role for HGF preventing heart fibrosis has been previously described in the cardiomyopathic Syrian hamster [15]. In this pathological model, HGF down-regulation in endothelial and interstitial cells triggers TGF- $\beta$ 1 up-regulation and interstitial fibrosis, whereas HGF treatment decreases TGF- $\beta$ 1 expression and suppressed fibrosis. We show here that under physiological conditions, HGF/Met signaling in cardiomyocytes is also essential to prevent fibrosis in adult heart. Furthermore, we demonstrate a negative correlation between Met activity and levels of pro-fibrotic factors such as TGF- $\beta$ 2 and TGF- $\beta$ 3. We also provide evidence that TGF- $\beta$  treatment leads to down-regulation of antioxidant enzymes in cultured cardiomyocytes, thus linking fibrosis and oxidative stress.

The accumulation of ROS found in  $\alpha$ -MHC*Met-KO* mutant hearts could be responsible for their dysfunction, based on the well-known link between oxidative stress and heart failure [51]. Consistently, the oxidation of several proteins might account for a reduced contractile heart capacity. For example, proteins regulating the release and uptake of Ca by the endoplasmic reticulum, such as SERCA, or proteins involved in the regulation of myofilaments can be inactivated through oxidation, which leads to a reduced contractile force [51,52]. In addition to the impact on contractile capacity, ROS accumulation can induce cardiomyocyte cell death [51]. The ability of HGF/Met to counteract SOD dysfunction in a neurodegenerative animal model of amyotrophic lateral sclerosis [53] and to regulate antioxidant defenses [40–42] has been reported. For example, *in vivo* hepatocyte-specific Met deletion unbalances ROS regulation and sensitizes cells to Fas-mediated apoptosis [40]. HGF treatment also leads to increased mitochondrial activity and mass in glioblastoma cells [54]. The link between increased ROS levels and mitochondrial

dysfunction in defective hearts is supported by other studies [51,52,55,56]. Therefore, it is tempting to speculate that mitochondrial functional alterations occurring in Met deficient cardiomyocytes could cause inappropriate antioxidant capability, leading to increased oxidative stress. It is also possible that the reduced contractile function observed in the  $\alpha$ -MHC*Met-KO* mice could be a consequence of the decrease in the number of mitochondria and/or their functionality [52] as they are critical to provide ATP for contraction.

The effects of Met inactivation in cardiomyocytes partially resemble heart defects in Sirt3-deficient mice [44]. These mice present heart fibrosis and ROS accumulation as a consequence of the decrease in catalase and SOD-2 expression in cardiomyocytes, but they also show cardiac hypertrophy [44]. The capability of Sirt3 and Met to prevent cardiac damage by suppressing ROS accumulation is in agreement with the lethality of SOD-2 deficient mice a few days after birth due to dilated cardiomyopathy, despite normal heart development [57]. It is also important to highlight that the contractile defect observed in the inducible HGF transgenic (tg) mice shares certain similarities to the one induced in  $\alpha$ -MHC*Met-KO* mice [21]. Because in these HGF tg mice, the endogenous Met receptor is downregulated, this might partially resemble our mouse model lacking a functional Met receptor in cardiomyocytes. However, different from the HGF tg mouse, HGF would not be over-expressed in our  $\alpha$ -MHC*Met-KO* mouse model.

Mechanistically, we provide *in vivo* and *in vitro* evidences supporting a role for p38 MAPK in the antioxidant effect of HGF/Met in cardiomyocytes. HGF increases SOD-2 and catalase expression and prevents their down-regulation by TGF- $\beta$  when p38 $\alpha$  MAPK signaling is intact. These findings are consistent with the previously reported role of p38 $\alpha$  MAPK as a mediator of SOD-2 and catalase expression in response to oxidative stress in MEFs [43]. These findings, together with recent studies on p38MAPK requirement for the survival effect of HGF in embryonic and cancer cells [58,59], unveil unexpected requirements of the p38MAPK pathway in cell regulation by receptor tyrosine kinases.

Our studies demonstrate a cell autonomous requirement of HGF/Met signaling in cardiomyocytes to ensure heart function in adult mice by preventing oxidative stress generation and ROS-induced damage. The control of TGF- $\beta$  production and p38 $\alpha$  MAPK activity by the HGF/Met axis appears to be essential for the maintenance of heart homeostasis. Indeed, p38 $\alpha$  MAPK appears to be an important mediator of HGF/Met actions, as it is required for the up-regulation of antioxidant enzymes. Notably, HGF not only regulates the production of TGF- $\beta$  in the heart, but also partially counteracts its effect on cardiomyocytes.

Our findings open a new perspective to further explore HGF/Met as putative diagnostic markers of individuals subjective to heart defects and therapeutic intervention to treat cases of idiopathic cardiomyopathies.

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## 585 Appendix A. Supplementary data

586 Supplementary data to this article can be found online at <http://dx>.  
 587 [doi.org/10.1016/j.bbadis.2013.08.008](http://doi.org/10.1016/j.bbadis.2013.08.008).

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