The calcineurin splicing variant CnAβ1 improves cardiac function following myocardial infarction without inducing hypertrophy

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ABSTRACT

Background – Calcineurin is a calcium-regulated phosphatase that plays a major role in cardiac hypertrophy. We previously described that alternative splicing of the calcineurin Aβ (CnAβ) gene generates the CnAβ1 isoform, with a unique C-terminal region that is different from the autoinhibitory domain present in all other CnA isoforms. In skeletal muscle, CnAβ1 is necessary for myoblast proliferation and stimulates regeneration, reducing fibrosis and accelerating the resolution of inflammation. Its role in the heart is currently unknown.

Methods and Results – We generated transgenic mice overexpressing CnAβ1 in postnatal cardiomyocytes under the control of the αMHC promoter. In contrast to previous studies using an artificially truncated calcineurin, CnAβ1 overexpression did not induce cardiac hypertrophy. Moreover, transgenic mice showed improved cardiac function and reduced scar formation following myocardial infarction, with reduced neutrophil and macrophage infiltration and decreased expression of proinflammatory cytokines. Immunoprecipitation and western blot analysis showed interaction of CnAβ1 with the mTORC2 complex and activation of the Akt/SGK cardioprotective pathway in a PI3K-independent manner. In addition, gene expression profiling revealed that CnAβ1 activated the transcription factor ATF4 downstream of the Akt/mTOR pathway to promote the amino acid biosynthesis program, reduce protein catabolism and induce the anti-fibrotic and anti-inflammatory factor GDF15 that protects the heart through Akt activation.

Conclusions – CnAβ1 shows a unique mode of action that improves cardiac function after myocardial infarction, activating different cardioprotective pathways without inducing

maladaptive hypertrophy. These features make CnAβ1 an attractive candidate for the development of future therapeutic approaches.

Keywords: myocardial infarction, signal transduction, amino acids, inflammation, hypertrophy

INTRODUCTION

Calcineurin (Cn) is a calcium-dependent serine/threonine phosphatase that regulates a wide variety of physiological and pathological processes.[1] Cn links changes in intracellular calcium to gene transcription by dephosphorylating the transcription factor NFAT (nuclear factor of activated T-cells), among others, and inducing its translocation to the nucleus.[1] It is composed of a catalytic subunit (CnA) and a regulatory subunit (CnB). Three different CnA isoforms are found in mammals: CnAα and CnAβ, which are ubiquitously expressed, and CnAγ, restricted to brain and testis. They all share a common structure including a phosphatase domain, a CnB-binding region, calmodulin-binding region and a C-terminal autoinhibitory domain. In its inactive state, the catalytic site in CnA is blocked by this autoinhibitory domain. In response to raised intracellular calcium, CnB and calmodulin activate CnA by inducing a conformational change that displaces the autoinhibitory domain and exposes the catalytic site. Artificial removal of the autoinhibitory domain renders CnA constitutively active and results in full NFAT activation.

In the heart, Cn participates in both cardiac development and disease. Cn plays a central role in the development of maladaptive but not physiological hypertrophy.[2] Transgenic mice overexpressing an artificially truncated, constitutively active form of CnAα that lacks the autoinhibitory domain show strong cardiac hypertrophy and develop heart failure within the first weeks of age, a response phenocopied by overexpression of a constitutively active form of NFAT.[3] In contrast, knockout mice lacking the phosphatase domain of CnAβ, the main CnA isoform in the heart, show smaller hearts at baseline and reduced hypertrophy in response to

pressure overload, angiotensin II or isoproterenol.[4] Similarly, overexpression of the endogenous Cn inhibitor RCAN1 prevents cardiac growth and ameliorates heart function.[5]

A protective role has also been proposed for Cn. Mice overexpressing constitutively active Cn show reduced apoptosis after ischemia/reperfusion,[6] whereas deletion of the phosphatase-encoding exon of CnAβ results in increased cell death and reduced cardiac function.[7]. However, the use of constitutively active Cn as a therapeutic agent for myocardial infarction is precluded by its strong induction of maladaptive cardiac hypertrophy.[3]

We have recently reported that an alternative splicing form of CnAβ (called CnAβ1) is induced by insulin growth factor 1 (IGF-1) and can on its own recapitulate the regenerative program elicited by IGF-1 in skeletal muscle.[8] This naturally occurring isoform lacks the autoinhibitory domain present in all other calcineurins and contains instead a unique C-terminal domain that has no similarity with any other known protein. Here we explore the effect of CnAβ1 overexpression in the heart.

METHODS

Full Methods can be found in the supplemental material.

Mice, Surgery and Cardiac Function Analysis

αMHC-CnAβ1 transgenic mice express CnAβ1 under the control of the alpha myosin heavy chain (αMHC) promoter, which allows post-mitotic cardiomyocyte-restricted expression. All operations were performed with the approval of the institutional ethics committee and the Home Office, UK. Ten-week-old αMHC-CnAβ1 transgenic mice (n=27) and age-matched wild type (WT) mice (n=27) were subjected to left coronary artery ligation and echocardiography analysis was performed 1 and 4 weeks later. Two-dimensional and M-mode images were obtained at the level of the papillary muscles using a Vevo-770 imaging system (VisualSonics). Percent fractional area contraction (%FAC), a parameter of systolic function, was calculated from the LV cross sectional area (%FAC=((LVEDA-LVESA)/LVEDA)x100); both LV end-diastolic area (LVEDA) and LV end-systolic area (LVESA) were measured by two-dimensional trace at the papillary muscle level, and three beats were averaged for each measurement. Hemodynamic analysis was performed under isoflurane anesthesia. A 1.4-Fr microtipped pressure transducer (SPR-839, Millar Instruments) was inserted through the right carotid artery and advanced into the LV for measurement of LV pressure by a blinded operator. Hemodynamic parameters were calculated from the recorded data using commercial software (PVAN3.2, Millar Instruments). Tau was calculated using Glantz method. For rapamycin experiments, adult WT and transgenic mice (n=5 for each condition) were injected i.p. daily for 4 days with 1 mg/Kg rapamycin (Biomol) diluted in 0.2 % (w/v) sodium carboxymethyl cellulose, 0.25 % (v/v) polysorbate 80.

Control mice received an equivalent i.p. injection of solvent alone. Three hours after the last injection, mice were sacrificed, hearts were excised and the LV was snap-frozen.

RNA extraction, qRT-PCR and microarrays

Total RNA was extracted and quantitative real time reverse transcribed-PCR (qRT-PCR) was performed as described using off-the-shelf Taqman probes (Applied Biosystems)[8, 9]. Analysis was performed using the ΔΔCt method with an 18S probe for normalization.

For microarray experiments, the different conditions were analyzed in triplicate using a MG_430A2.0 oligonucleotide-based microarray (Affymetrix).[8] Data were analyzed using Genespring 7.3 (Silicon Genetics). Each chip was normalized to the 50th percentile and all samples were normalized to the WT uninjured samples. Those genes that had a raw expression >10 in 4 out of 6 conditions, flag present or marginal in 6 out of 18 samples and a difference in normalized expression < or >1.7-fold were selected for each condition. Gene Ontology (GO) analysis was performed as previously described.[8, 9]

Western blot

Tissue lysates were separated in SDS-PAGE gels, transferred to nitrocellulose blots and blocked with 5 % non-fat dry milk in TBS (10 mM Tris-HCl pH7.4, 150 mM NaCl). Blots were incubated with primary antibodies overnight, followed by appropriate HRP-labeled secondary antibodies. Mouse monoclonal anti-CnAβ1 has been previously described.[8] Antibodies against phospho-PDK1, phospho-Akt, Akt, phospho-SGK, SGK, p-eIF2α, eIF2α, p-GCN2 and p-Bad were purchased from Cell Signaling. Anti-ATF4 was obtained from Abcam and anti-NFATc1

was from Santa Cruz. Anti-Stag2 was a kind gift from Dr. Jose Luis Barbero (Centro de Investigaciones Biológicas, Madrid, Spain).

RESULTS

CnAβ1 does not induce cardiac hypertrophy

To examine the effect of CnAβ1 overexpression in the heart, we developed transgenic mice expressing CnAβ1 under the control of the α myosin heavy chain promoter (αMHC-CnAβ1 mice), which allows post-natal overexpression of CnAβ1 specifically in cardiac myocytes. Expression of both CnAβ1 mRNA and protein were induced in transgenic hearts compared to wild type (WT) mice (Fig. 1A, 1B). No significant difference in cardiac function, heart weight to body weight ratio or ventricular dilation was detected in transgenic mice (Fig. 1C-E, Table 1). In addition, adult cardiomyocytes isolated from αMHC-CnAβ1 mice displayed similar cell surface area to those from WT mice (Fig. 1F). Analysis of different heart failure and remodeling markers by qRT-PCR showed no difference in brain natriuetic peptide (BNP), α-skeletal actin (Acta1) or β-myosin heavy chain (β-MHC) expression in αMHC-CnAβ1 mice (Fig. 1G). Similarly, no changes were found for the fibroblast marker Thy1/CD90, collagen 1α1 (Col1a1) or lysyl oxidase (Lox), the enzyme responsible for collagen 1 and collagen 3 cross-linking (Fig. 1H). Together, these results indicate that CnAβ1 overexpression in the heart does not induce cardiac hypertrophy, fibrosis or remodeling, in contrast with the artificially truncated CnA form.[3]

CnAβ1 improves cardiac function after myocardial infarction

To determine the effect of CnAβ1 on the response to cardiac injury, we induced myocardial infarction by permanently ligating the left coronary artery in both WT and transgenic mice. αMHC-CnAβ1 mice showed improved survival after infarction compared to WT (Fig. 2A),

although the difference did not reach statistical significance. Transgenic mice had significantly reduced scar area and collagen deposition 28 days post-injury (Fig. 2B, 2C). In addition, whereas WT animals developed compensatory cardiomyocyte hypertrophy after infarction, transgenic cardiomyocytes showed significantly smaller cross-sectional area than WT cells (Fig. 2D). Echocardiography confirmed significant dilation of the left ventricle in WT mice from 7 days after infarction, accompanied by a decrease in systolic function as determined by fractional area change (% FAC, Table 1). In contrast, αMHC-CnAβ1 mice showed smaller end systolic and diastolic areas and a significantly higher % FAC (1.5-fold). Similar results were found at 28 days post-injury (p=0.069). In order to confirm these results, we measured hemodynamic parameters using an invasive catheter 28 days after the surgery. WT mice presented a significant increase in end diastolic pressure (LVEDP), together with reduced contraction and relaxation capacities, as measured by dP/dT_{max}, dP/dT_{min} and Tau g. Overexpression of CnAβ1 significantly prevented this deterioration of contractile and relaxation functions (Table 1). Together, these results show that CnAβ1 exerts a beneficial effect on the heart in the context of myocardial infarction.

Reduced fibrosis and accelerated resolution of inflammation in αMHC-CnAβ1 mice

We analyzed different fibrosis and inflammation parameters by qRT-PCR in the infarct region, the border zone and the myocardial area remote to the infarct. The cardiac fibroblast proliferation marker Thy1[10] showed a strong induction in the infarct region and the border zone by 7 days post-injury in WT mice (Fig. 3A) that was significantly lower in αMHC-CnAβ1 animals. Both Col1a1 and Lox were strongly induced in the infarct region and border zones in WT mice, particularly 7 days after infarction when the scar is being formed (Fig. 3B, 3C). CnAβ1 overexpression significantly decreased the expression of these markers in the infarct region 28

days after surgery. In the border zone a reduction could already be seen by 7 days. Expression of fibrotic markers was also detected in the remote myocardial area, indicating the induction of interstitial fibrosis, although the expression was much lower than in the infarct region (Fig. 3A-C). Transgenic mice showed lower levels of Col1a1 and Lox in this area as well (Fig. 3B, 3C).

We next analyzed the expression of different leukocyte population markers and inflammatory mediators. To determine the presence of neutrophils and macrophages, we measured the expression of granzyme B (Gzmb) and CD68 respectively. We observed robust expression of both markers at 7 days post-injury in the infarct region and border zone of both WT and transgenic mice (Fig. 3D, 3E). However whereas expression of both Gzmb and CD68 were almost normalized in the border zone at 28 days in both groups, elevated expression persisted in the infarct region in WT mice. Notably levels of these markers were significantly lower in the infarct region of transgenic mice suggesting a faster resolution of inflammation in these mice compared to WT. Similarly we observed a strong induction of the pro-inflammatory cytokine interleukin 6 (IL-6) in the infarct region and border zone of WT and transgenic mice 7 days post-injury (Fig. 3F). Whereas elevated IL-6 levels persisted at 28 days post-injury in WT mice, αMHC-CnAβ1 hearts showed significantly lower IL-6 expression. Collectively, these results indicate that CnAβ1 decreases fibrosis and accelerates the resolution of inflammation following myocardial infarction.

Transcription profile of wild type and transgenic mice after myocardial infarction

To gain insight into the molecular mechanisms underlying the beneficial effects of CnAβ1, we analyzed the transcription profile of uninjured left ventricle samples and myocardial samples from the area remote to the infarct 7 and 28 days post-injury using oligonucleotide microarrays.

As expected, we observed an increase in extracellular matrix and inflammation genes in WT mice after infarction, together with a decrease in mitochondrial metabolism genes (for detailed explanation, please see Suppl. Results, Suppl. Fig. 1, Suppl. Tables 1-20).

In this context, we looked for genes that showed differential expression (>1.7-fold) between WT and transgenic mice at each time point. In the absence of injury, αMHC-CnAβ1 mice showed decreased expression of genes involved in caspase activation and the response to stress (Suppl. Tables 23-24) but no clear pattern among the upregulated genes (Suppl. Tables 21-22). Seven days after infarction, we found a robust increase in activating transcription factor 4 (ATF4) targets, amino acid biosynthesis and cyclin D2-associated processes in αMHC-CnAβ1 mice (Suppl. Tables 27-28) together with reduced expression of genes involved in cell proliferation, amino acid catabolism and fatty acid metabolism (Suppl. Tables 31-32, Suppl. Results, Suppl. Fig. 2). Four weeks after infarction, the upregulation of amino acid biosynthesis genes persisted (Suppl. Tables 35-36) and was accompanied with a decrease in genes involved in rhythmic processes (Suppl. Tables 39-40). Together, these results suggest that CnAβ1 partially prevents the molecular processes associated with myocardial infarction and that it specifically targets the amino acid biosynthesis program.

CnAβ1 activates ATF4

To validate the microarray results, we first analyzed ATF4 mRNA expression by qRT-PCR and found that it was significantly induced in the infarct region and the remote area of αMHC-CnAβ1 hearts compared to WT at 7 days post-injury (Fig. 4A). Since ATF4 expression is mainly controlled at the translational level[11], we performed western blot analysis and observed an upregulation of ATF4 protein in myocardial samples from transgenic animals (Fig. 4B).

Immunofluorescence analysis at 7 days post-injury showed that ATF4 was principally expressed by cardiomyocytes, although it could also be detected in some non-muscle cells (Fig. 4C). qRT-PCR analysis of the ATF4 targets ATF5 and asparagine synthetase (Asns) confirmed a strong upregulation of both molecules in the remote area of αMHC-CnAβ1 mice 7 days post-injury (Fig. 4D, 4E). In addition, we observed that expression of the cardioprotective factor growth differentiation factor 15 (GDF15), which was also identified in the arrays (Suppl. Table 25) and is an ATF4 target[12], was significantly increased in the remote area of transgenic mice 7 days after infarction (Fig. 4F). This induction was also seen in the infarct region and in uninjured hearts.

CnAβ1 activates protective signaling pathways in the heart

To identify the signaling pathways mediating the protective action of CnAβ1, we compared the proteomic profile of WT and transgenic hearts using antibody microarrays. CnAβ1-induced changes in protein phosphorylation and expression were similar in uninjured samples and remote myocardium 7 days post-injury (p=0.0015, non-parametric correlation). In both uninjured and infarcted hearts CnAβ1 activated components of the Akt pathway (Suppl. Tables 41-43), including PRAS40 and mTOR. Western blot analysis revealed increased phosphorylation of PDK1, Akt, SGK, PRAS40 and mTOR in response to CnAβ1 overexpression, confirming the microarray results, and inactivation of the proapoptotic protein Bad (Fig. 5A). In addition, transfection of WT neonatal cardiomyocytes with CnAβ1 siRNA reduced Akt phosphorylation (Fig. 5B), indicating that CnAβ1 is both necessary and sufficient for Akt activation. Only a modest increase in the amount of nuclear NFAT was detected in αMHC-CnAβ1 mice (Fig. 5C). Similarly, we found no significant differences in CnAα, CnAβ2, CnB or calmodulin expression

between WT and αMHC-CnAβ1 mice (Suppl. Fig. 3B, 3C, 3E). Moreover, we found no significant increase in Cn enzymatic activity or in the expression of the NFAT-regulated gene Rcan1/Mcip1/Dscr1 (Suppl. Fig. 3D, 3F), suggesting that the NFAT signaling pathway was not activated in αMHC-CnAβ1 hearts.

To determine how the Akt pathway is activated by CnAβ1, we used HEK293T cells, which can be efficiently transfected. Overexpression of CnAβ1 led to a mild increase in Akt activation (Fig. 5D), compared to both CnAβ2 and a truncated constitutively active CnAβ form lacking the autoinhibitory and unique domains (CnAβ*). Importantly, inhibition of PI3K activity with LY294002 blocked Akt activation in all conditions except in the presence of CnAβ1, suggesting that CnAβ1 was activating the Akt pathway below PI3K. To explore whether CnAβ1 could interact with mTOR complex 2 (mTORC2), we transfected HEK293T cells with GFP-containing chimeras linked to either full CnAβ1 and CnAβ2 proteins (Fig. 6A) or to their respective C-terminal domains (Fig. 6B). Both Rictor and mTOR, components of the mTORC2 complex, strongly co-immunoprecipitated with GFP-CnAβ1 (Fig. 6A), whereas only a weak band could be seen in the GFP-CnAβ2 lane. Furthermore, a GFP chimera linked to the C-terminal domain of CnAβ1 was sufficient to co-immunoprecipitate Rictor and mTOR (Fig. 6B), in contrast to a construct carrying GFP linked to the autoinhibitory domain of CnAβ2, indicating that the unique C-terminal domain of CnAβ1 is both necessary and sufficient to interact with mTORC2. We confirmed the interaction between endogenous CnAβ1 and Rictor *in vivo* by immunoprecipitation in WT uninjured hearts (Fig. 6C). Moreover, we found that both endogenous and overexpressed CnAβ1 interact with CnB (Fig. 6A, 6D), suggesting that both molecules exist as a dimer.

Although ATF4 was initially described as a stress-inducible factor[13], it can also be activated by the PI3K/Akt/mTOR pathway in response to growth factors and cytokines.[14, 15] Whereas we did not detect activation of stress response mediators eIF2α or eIF2α kinases PKR, PERK or Gcn2 in αMHC-CnAβ1 mice (not shown), inhibition of mTORC1 activity with rapamycin reduced ATF4 induction by CnAβ1 (Fig. 7A). Similarly, rapamycin partially prevented the induction of ATF4 targets Asns, ATF5 and GDF15 down to 50 %, without affecting CnAβ1 or CnAβ2 expression (Fig. 7B-7F). To explore whether activation of the mTOR pathway was also mediating the cardioprotective action of CnAβ1, we studied the response of neonatal cardiomyocytes to hypoxia. Exposure of WT cardiomyocytes to 1 % oxygen decreased cell viability, as shown by an increase in lactate dehydrogenase (LDH) released to the culture medium, whereas transgenic cells were unaffected (Fig. 7G). Although inhibition of PI3K activity with LY294002 had no effect on the protective action of CnAβ1, blockade of mTOR with rapamycin resulted in similar loss of cell viability in WT and transgenic cardiomyocytes.

DISCUSSION

We report here that the physiological, naturally occurring, calcineurin isoform CnAβ1 improves cardiac function after myocardial infarction, reducing inflammation and scar formation. Functional improvement is already evident one week after infarction, suggesting that CnAβ1 may have a cardioprotective action. This cardioprotective effect is reproduced in culture, where CnAβ1 protects cardiomyocytes from cell death induced by hypoxia. In the heart, PDK1 and Akt mediate the beneficial effects of ischemic preconditioning.[16, 17] and SGK mediates the cardioprotective action of IGF-1.[18, 19] We had previously reported that CnAβ1 is necessary for the activation of the Akt pathway in proliferating myoblasts.[8] Here we now show that CnAβ1 is not only necessary but also sufficient to elicit this protective mechanism. Our data suggest that both mTORC2 and PDK1 are involved in the activation of the Akt/SGK pathway by CnAβ1, since both Ser^{473} and Thr^{308} in Akt are more phosphorylated in CnAβ1-overexpressing mice. Activation of Akt by CnAβ1 is insensitive to PI3K inhibition, suggesting that CnAβ1 enters the Akt pathway below PI3K. After exploring different components of the pathway, we found that CnAβ1 interacts with the mTORC2 complex. Moreover we demonstrated that the unique C-terminal domain of CnAβ1 mediates this interaction and is necessary for Akt activation by CnAβ1. Neither CnAβ2 nor a truncated constitutively active CnAβ with no autoinhibitory or unique domains show this ability, indicating that the alternative C-terminal domain confers unique properties to CnAβ1 that are not shared by other Cn isoforms. Although chronic strong activation of Akt induces maladaptive cardiac hypertrophy,[20] we see no hypertrophy in αMHC-CnAβ1 mice. It is likely that the levels of Akt activation in these mice are below those required

for the induction of cardiac hypertrophy, as shown for other mouse models in which mild chronic Akt activation promotes survival without inducing hypertrophy.[21]

The protective activity of CnAβ1 is reminiscent of the IGF-1Ea isoform, which improves cardiac function by activating the PDK1/SGK axis with no cardiac hypertrophy.[18] IGF-1Ea has anti-inflammatory and anti-fibrotic properties that were also mimicked by CnAβ1, which decreased the presence of neutrophils and macrophages, the expression of inflammatory cytokines and fibrotic markers and reduced scar formation. The anti-inflammatory and anti-fibrotic actions of CnAβ1 may be mediated at least in part by GDF15, a member of the transforming growth factor-β (TGF-β) family that inhibits macrophage activation. GDF15 is produced by cardiomyocytes in both hypertrophic and ischemic hearts[22, 23] and has a protective anti-apoptotic action mediated by Akt that limits heart damage after infarction, reduces scar formation and improves cardiac function.[23] In addition, it attenuates cardiac hypertrophy, limits ventricular dilation and reduces heart failure in different mouse models.[22]

Our data identify ATF4 as a potential mediator of the cardioprotective actions of CnAβ1. ATF4 protects the cell from stress by activating the amino acid biosynthesis program and inducing expression of anti-oxidant genes such as glutathione[24] and protective growth factors like GDF-15.[12, 25] ATF4 is principally controlled at the translational level in response to endoplasmic reticulum (ER) stress or amino acid starvation[13, 25]. However, we observed no activation of the associated stress-induced kinases PERK, PRK or Gcn2, no phosphorylation of eIF2α and no induction of a gene expression pattern indicative of ER stress in CnAβ1-overexpressing mice. Alternatively, ATF4 can be activated by anabolic stimuli, like insulin, IGF-1 and fetal serum, through the mTORC1 complex to enhance amino acid uptake and biosynthesis and to decrease protein catabolism.[14] We found that induction of ATF4 and its targets by CnAβ1

was abolished by rapamycin injection, suggesting that CnAβ1 activates ATF4 through mTORC1 independently of the ER stress and the amino acid starvation response. Given that CnAβ1 expression is induced by IGF-1[8], it is reasonable that both molecules share a common pathway for the activation of amino acid anabolism through ATF4, as they do for the blockade of protein catabolism and muscle atrophy through FoxO inhibition.[8] Collectively, our data support a role for ATF4 and amino acid biosynthesis in cardioprotection and the activation of this transcription factor through Akt independently of ER stress.

In conclusion, activation of CnAβ1 in the heart elicits a cardioprotective pathway that contributes to decrease scar formation and to improve cardiac function after myocardial infarction. Although some of these actions are shared by the truncated CnA[6, 26], artificial CnA activation has the major disadvantage of rapidly inducing cardiac hypertrophy and heart failure[3], making it unsuitable for therapeutic purposes. In contrast, the naturally active CnAβ1 isoform protects the myocardium from the detrimental effects of ischemic injury without inducing hypertrophy. These features make CnAβ1 an attractive candidate for the development of future therapeutic approaches.

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DISCLOSURES

The authors have nothing to disclose.

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FIGURE LEGENDS

Figure 1. Cardiac overexpression of CnAβ1 does not induce cardiac hypertrophy. A, B, Hearts were excised from adult wild type (WT) and αMHC-CnAβ1 mice and CnAβ1 mRNA and protein expression was analyzed by qRT-PCR (**A**) and western blot (**B**), respectively. **C, D,** Left ventricular end diastolic area (LVEDA, **C**) and fractional area change (% FAC, **D**) were measured in WT and transgenic mice using echocardiography and expressed as average ±SE. **E**, Heart and body weight of WT and αMHC-CnAβ1 mice was determined at different ages and represented as average ratio (mg/g) ±SE. **F**, Cardiac myocytes were isolated from adult WT and αMHC-CnAβ1 hearts and the average cell area ±SE was quantified. **G, H,** mRNA expression of the indicated heart failure (**G**) and fibrosis (**H**) markers was determined by qRT-PCR and expressed as fold induction ±SE over WT. ***p<0.0005.

Figure 2. CnAβ1 reduces cardiac scar fibrosis after myocardial infarction. A, Myocardial infarction (MI) was induced by permanent ligation of the left coronary artery in wild type (WT) and transgenic mice and survival was determined over a 28 day period. **B, C,** Infarcted hearts were isolated 28 days after surgery, processed for histological analysis and stained with Sirius red for determination of collagen content. Bar, 100 μm. Scar area (**C**) was quantified and expressed as percentage over total area ±SE. *p<0.05. **D,** Cardiomyocyte cross-sectional area (CSA) was quantified in uninjured and infarcted mice 28 days post-injury and expressed as fold induction ±SE over uninjured WT mice. *p<0.05 infarcted vs uninjured; #p<0.05 αMHC-CnAβ1 vs WT.

Figure 3. Reduced expression of fibrosis and inflammation markers in αMHC-CnAβ1 mice. A-H, Expression of different fibrosis and inflammation markers was analyzed by qRT-PCR in myocardial samples from the infarct region (Infarct), the infarct border zone (Border) and the area remote to the infarct (Remote) 7 and 28 days after infarction. **A**, fibroblast proliferation marker Thy1; **B**, collagen 1a1 (Col1a1); **C**, lysyl oxidase (Lox), which crosslinks collagen I and III fibers; **D**, neutrophil marker granzyme B (Gzmb); **E**, macrophage marker CD68; **F,** pro-inflammatory cytokine IL-6. Results are expressed as fold induction over the WT uninjured values ±SE. *p<0.05 WT uninjured vs. WT infarcted; #p<0.05 WT vs. αMHC-CnAβ1 for each experimental condition.

Figure 4. CnAβ1 activates ATF4 and the amino acid biosynthesis program after myocardial infarction. A, Expression of activating transcription factor 4 (ATF4) mRNA was determined by qRT-PCR in the infarct, border and remote areas 7 and 28 days post-injury. Results are expressed as fold induction ±SE over LV samples from WT uninjured hearts. *p<0.05 WT uninjured vs. WT infarcted; #p<0.05 WT vs. αMHC-CnAβ1 for each experimental condition. **B**, The expression of ATF4 in the myocardial area remote to the infarct was analyzed by western blot and compared to uninjured WT and transgenic LV samples. β-Catenin shows equal protein loading. **C,** ATF4 localization (green) was determined by immunofluorescence in the myocardial area remote to the infarct. Cardiomyocytes were counterstained with α-sarcomeric actin (red) and nuclei with DAPI (blue). Bar, 50 μm. **D-F**, Expression of ATF5, asparagine synthetase (Asns) and growth differentiation factor 15 (GDF15) was determined by qRT-PCR as in (A).

Figure 5. CnAβ1 activates the Akt signaling pathway in the heart. A, PDK1, Akt, SGK, PRAS40, mTOR, GSK3β and Bad phosphorylation and expression was analyzed by western blot in WT and αMHC-CnAβ1 hearts. **B**, Neonatal rat cardiomyocytes were transfected with Luciferase (Control) or CnAβ1 siRNAs and Akt phosphorylation analyzed by western blot. **C**, NFAT localization was determined by western blot in nuclear and cytoplasmic extracts. Total Akt and Stag2 show equal protein load. **D**, HEK293T cells were transfected with pcDNA3-derived vectors expressing CnAβ1, CnAβ2, a truncated constitutively active form of CnAβ with no autoinhibitory domain (CnAβ*), or empty pcDNA3 as a control. Cells were treated with the PI3K inhibitor LY294002 (15 μM) or DMSO (1:1000; negative control) for 16 h and Akt phosphorylation was determined as in (C).

Figure 6. CnAβ1 interacts with the mTORC2 complex. A, B, HEK293T cells were transfected with expression plasmids encoding GFP linked to full CnAβ1 or CnAβ2 proteins (GFP-CnAβ1 and GFP-CnAβ2; A), to CnAβ1 unique C-terminal domain (GFP-CnAβ1-Cter; B) or to CnAβ2 autoinhibitory domain (GFP-CnAβ2-Cter; B). Proteins interacting with Rictor or GFP-containing chimeras were detected by immunoprecipitation followed by western blot. **C,** Rictor was immunoprecipitated from WT heart lysates and CnAβ1 was detected by western blot. **D**, Interaction between CnAβ1 and CnB was detected in WT and αMHC-CnAβ1 hearts by immunoprecipitation followed by western blot.

Figure 7. The induction of ATF4 by CnAβ1 is mediated by mTOR. A, WT and αMHC-CnAβ1 mice were injected i.p. daily with 1 mg/Kg rapamycin (+) or vehicle (-) for four days.

Mice were sacrificed 3 h following the last injection and the expression of ATF4 in the LV was analyzed by western blot. **B-F**, Expression of CnAβ1, CnAβ2 and the ATF4 targets Asns, ATF5 and GDF15 was analyzed in these hearts by qRT-PCR. Results are expressed as fold induction over WT uninjured ±SE. *p<0.05, ***p<0.0005 WT vs. αMHC-CnAβ1; #p<0.05 rapamycin vs vehicle (Control). **G**, Neonatal cardiomyocytes from WT and αMHC-CnAβ1 mice were exposed to 1 % oxygen in the presence of 15 μM LY294002, 1 μM rapamycin or 1:1000 DMSO (Control) and cell death was quantified by measuring the release of lactate dehydrogenase (LDH) to the culture medium. Results are expressed as fold induction ±SE over normoxic untreated cells for each mouse line. *p<0.05, *p<0.005, ***p<0.0005 hypoxia vs normoxia; #p<0.05, ###p<0.0005 WT vs. αMHC-CnAβ1. **H**, Schematic summarizing the activation of cardioprotective signaling by CnAβ1 in the heart.

TABLES

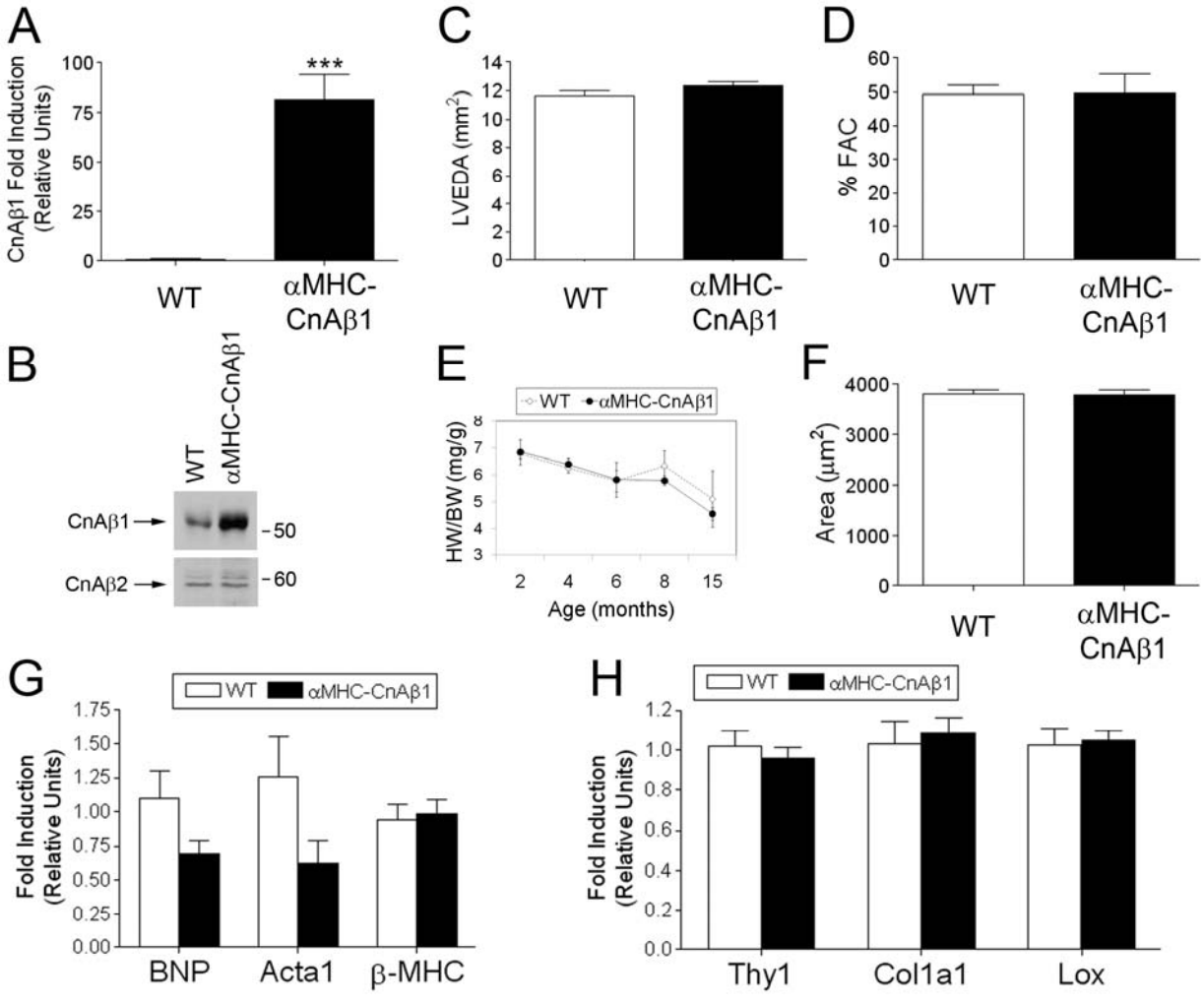
Echocardiography	Uninjured				MI 7d			
	WT		αMHC-CnAβ1		WT		αMHC-CnAβ1	
LVPW (mm)	0.65	±0.01	0.64	±0.02	0.59	±0.06	0.72	±0.03*
LVESA (mm^2)	5.95	±0.42	6.22	±0.23	17.95	±2.42*	12.63	±1.44*
LVEDA (mm^2)	11.65	±0.34	12.33	±0.28	21.89	±2.47*	17.67	±1.25*
% FAC	49.56	±2.76	49.57	±1.39	19.8	±2.08*	29.81	±3.12*†
n	14		15		9		8	

Echocardiography	Uninjured				MI 28 d			
	WT		αMHC-CnAβ1		WT		αMHC-CnAβ1	
LVPW (mm)	0.65	±0.01	0.64	±0.02	0.63	±0.08	0.68	±0.05
LVESA (mm^2)	5.95	±0.42	6.22	±0.23	17.8	±2.68*	13.2	±1.49*
LVEDA (mm^2)	11.65	±0.34	12.33	±0.28	21.77	±2.21*	18.66	±1.31*
% FAC	49.56	±2.76	49.57	±1.39	20.22	±4.17*	30.29	±2.96*
n	14		15		5		7	

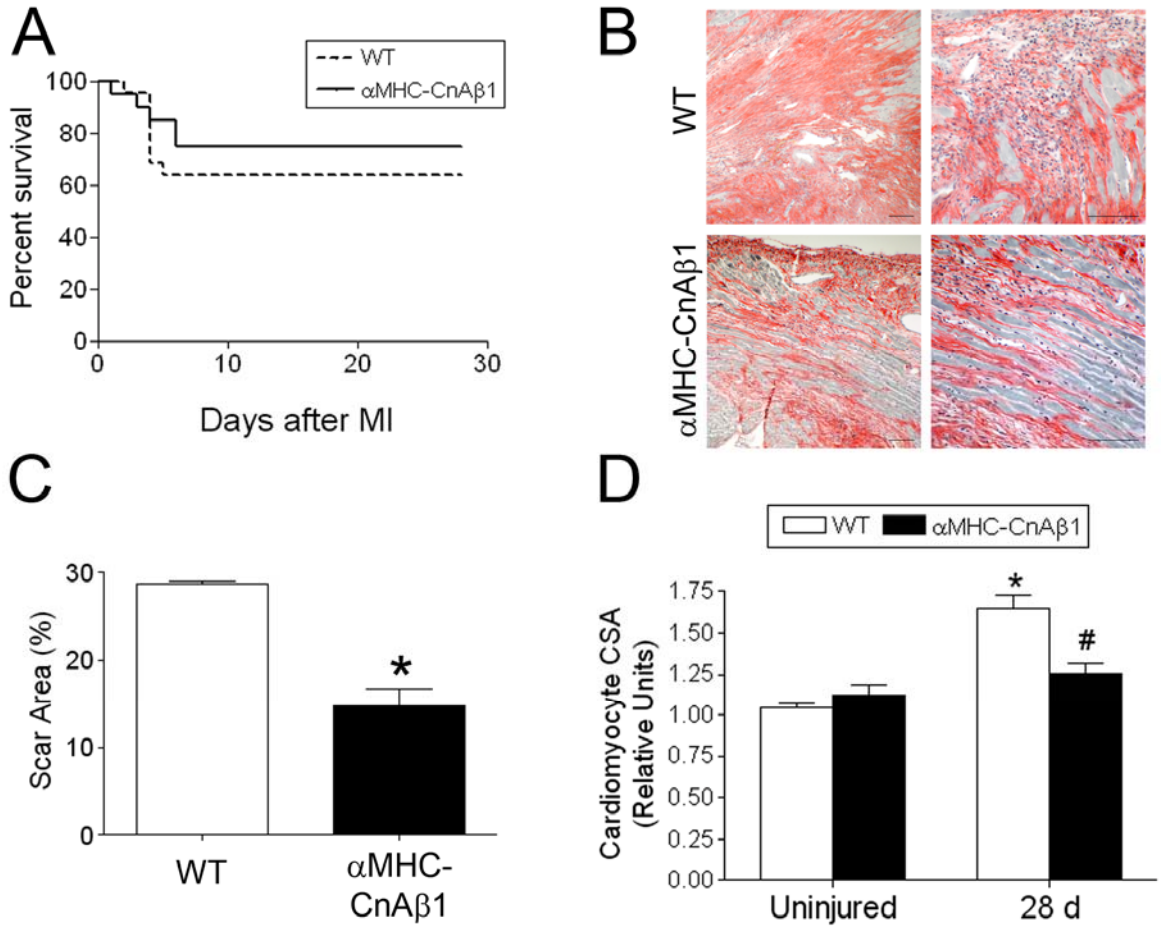
Hemodynamics	Uninjured				MI 28 d			
	WT		αMHC-CnAβ1		WT		αMHC-CnAβ1	
HR (bpm)	533	±20	547	±35	512	±31	536	±16
LVEDP (mmHg)	5.82	±0.88	8.26	±1.99	13.94	±3.1*	7.06	±1.02†
dP/dT_{max} (mmHg/s)	9929	±668	11439	±1523	7219	±709*	8988	±352*†
dP/dT_{min} (mmHg/s)	-9804	±657	-10092	±886	-5896	±723*	-7872	±490*†
Tau g (ms)	8.63	±0.70	8.02	±0.41	15.96	±2.31*	10.67	±0.71*†
n	6		3		5		7	

Table 1. CnAβ1 improves cardiac function after myocardial infarction. Ligation of the left coronary artery was performed in wild type (WT) and αMHC-CnAβ1 mice and echocardiography analysis was carried out 7 and 28 days later. Hemodynamic parameters were measured by catheterization 28 days after the surgery. Average ± SEM is shown. *p<0.05 infarcted vs uninjured; †p<0.05 transgenic vs WT, parametric unpaired t-test.

FIGURES



Felkin et al., Fig. 1



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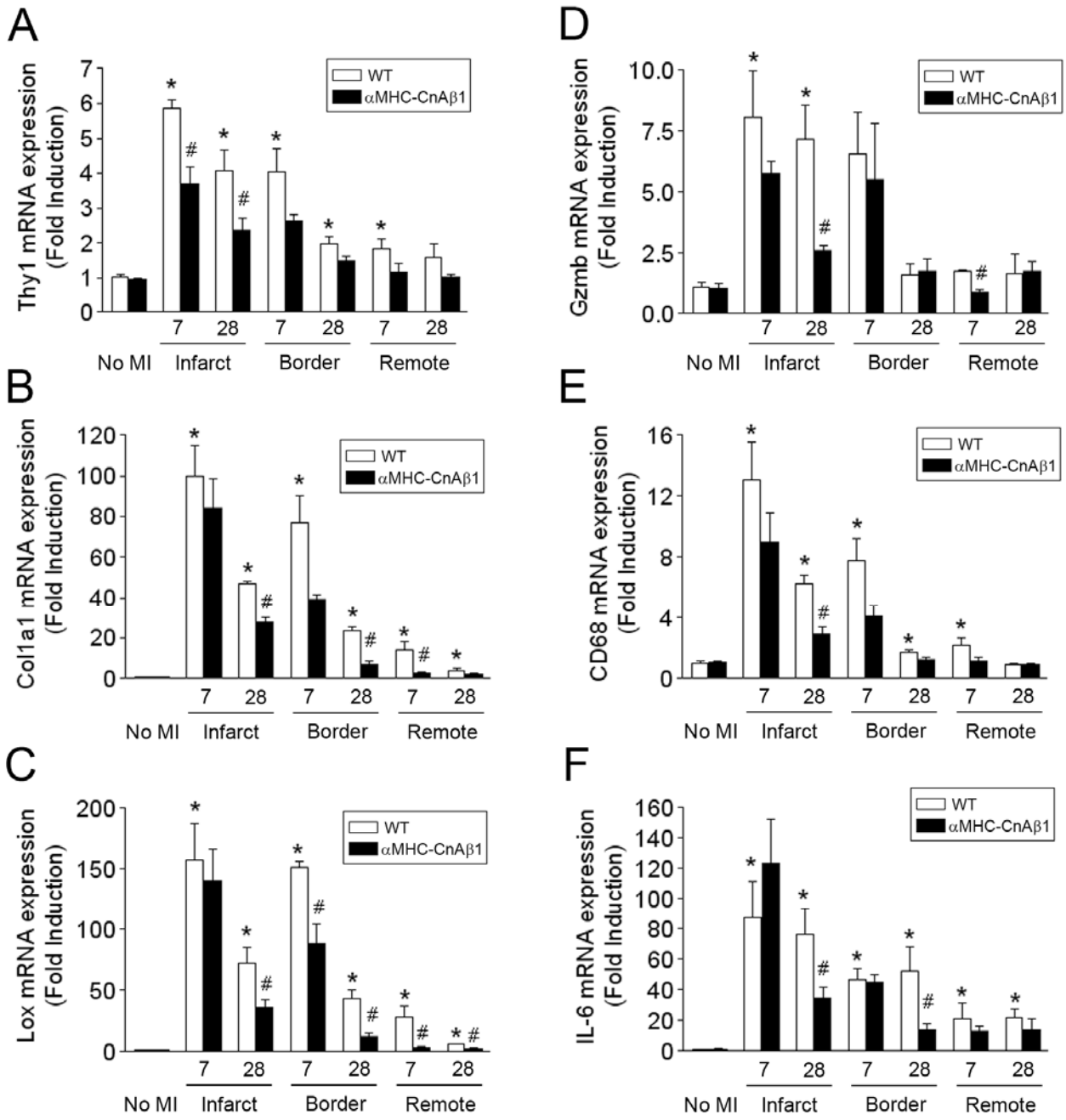

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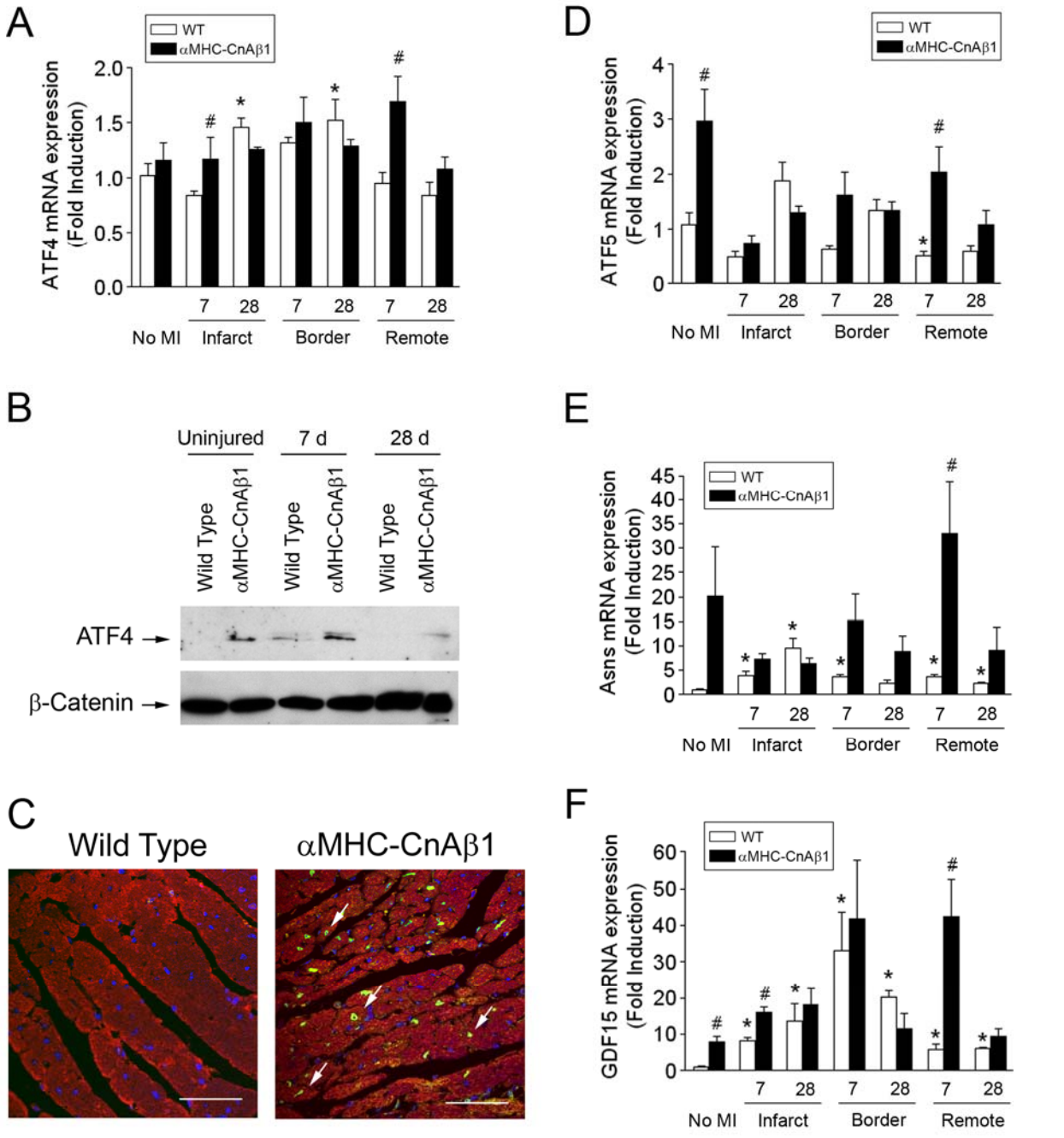

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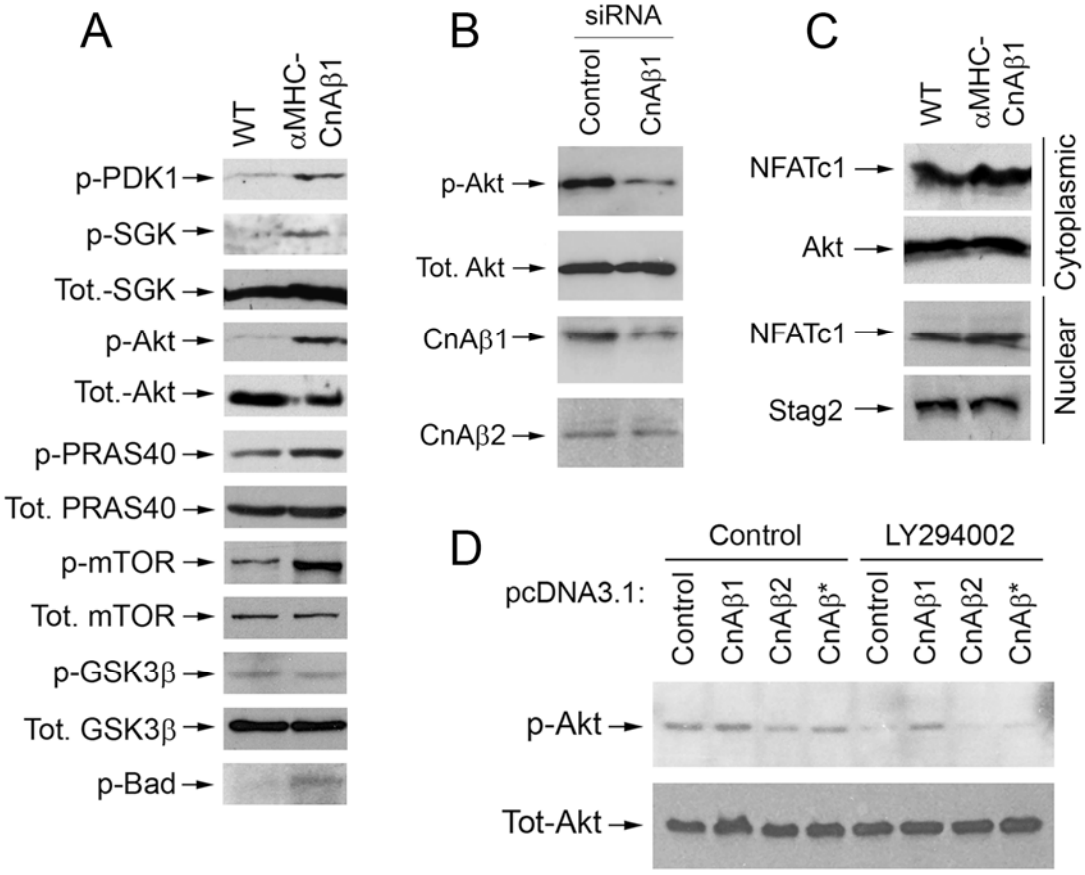

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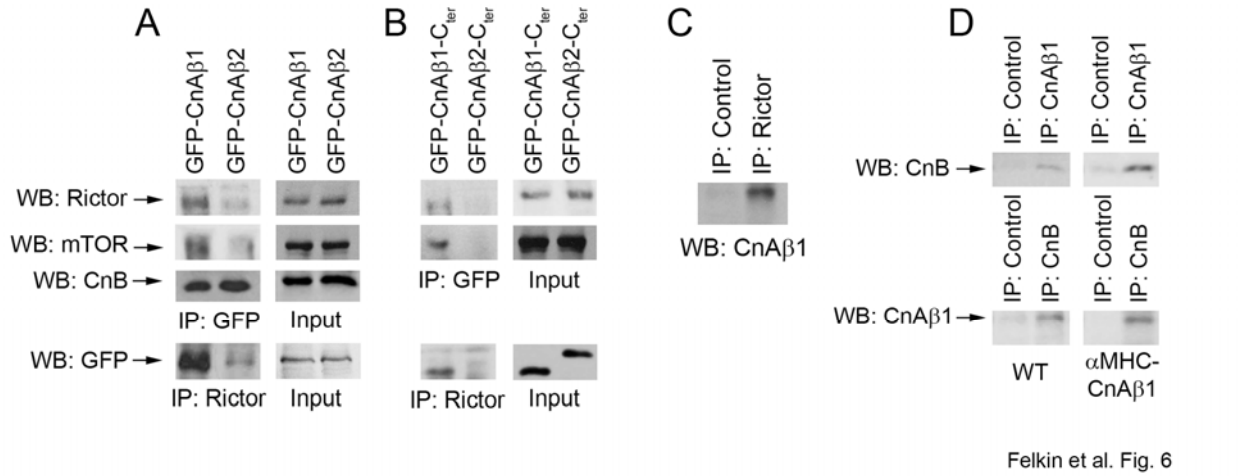

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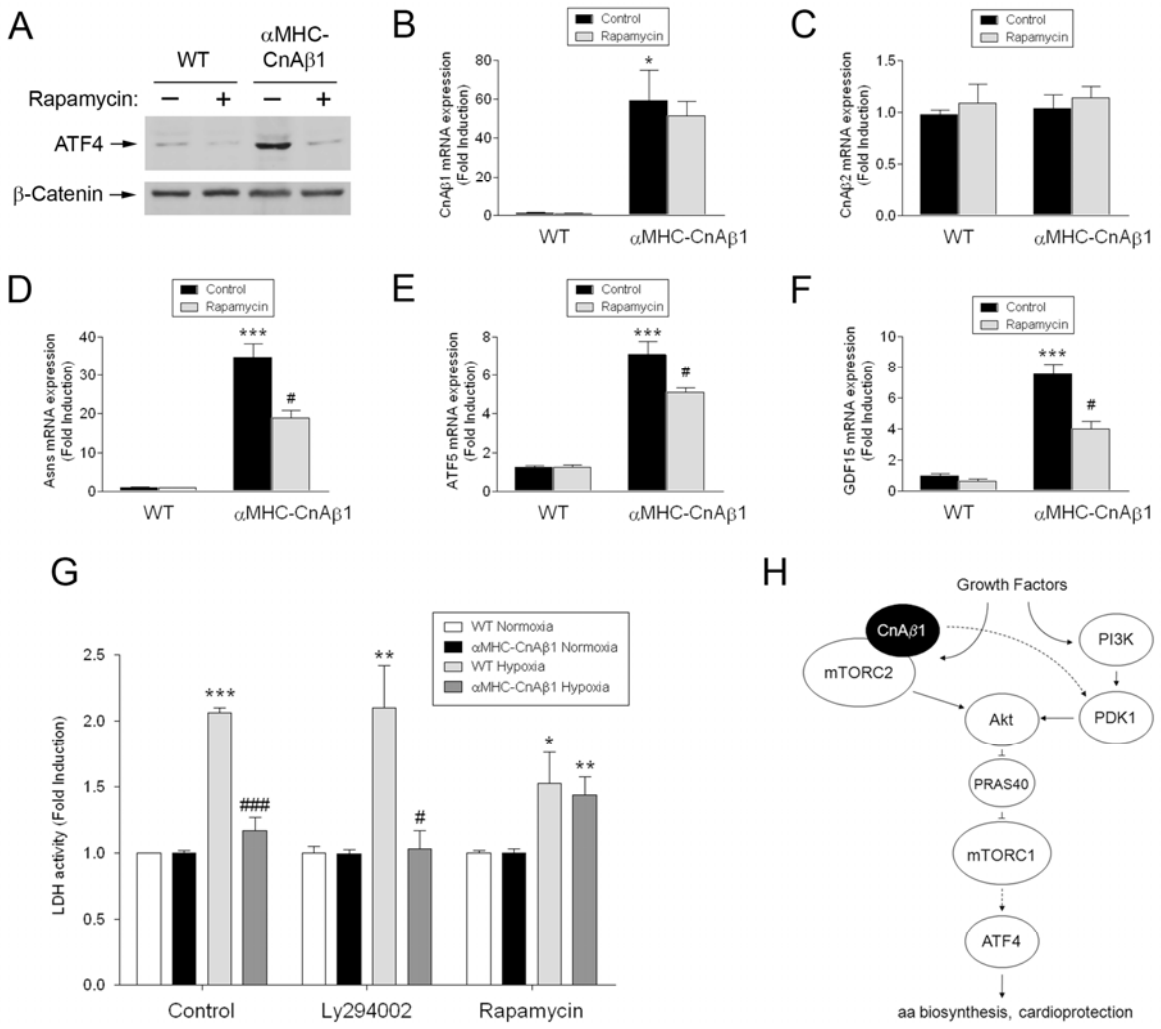

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