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Telethonin deficiency is associated with maladaptation to biomechanical stress in the mammalian heart

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Disclosures
None

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Abstract

Rationale—Telethonin (also known as titin-cap or t-cap) is a 19 kDa Z-disk protein with a unique β-sheet structure, hypothesized to assemble in a palindromic way with the N-terminal portion of titin and to constitute a signalosome participating in the process of cardio-mechanosensing. In addition, a variety of telethonin mutations are associated with the development of several different diseases; however, little is known about the underlying molecular mechanisms and telethonin's *in vivo* function.

Objective—Here we aim to investigate the role of telethonin *in viv*o and to identify molecular mechanisms underlying disease as a result of its mutation.

Methods and Results—By using a variety of different genetically altered animal models and biophysical experiments we show that, contrary to previous views, telethonin is not an indispensable component of the titin-anchoring system, nor is deletion of the gene or cardiac specific overexpression associated with a spontaneous cardiac phenotype. Rather, additional titin-anchorage sites, such as actin-titin crosslinks via α-actinin, are sufficient to maintain Z-disk stability despite the loss of telethonin. We demonstrate that a main novel function of telethonin is to modulate the turnover of the pro-apoptotic tumor suppressor p53 after biomechanical stress in the nuclear compartment, thus linking telethonin, a protein well known to be present at the Z-disk, directly to apoptosis ("mechanoptosis"). In addition, loss of telethonin mRNA and nuclear accumulation of this protein is associated with human heart failure, an effect which may contribute to enhanced rates of apoptosis found in these hearts.

Conclusions—Telethonin knockout mice do not reveal defective heart development or heart function under basal conditions, but develop heart failure following biomechanical stress, owing at least in part to apoptosis of cardiomyocytes, an effect which may also play a role in human heart failure.

Keywords

Genetics; Mechanosensation; Mechanotransduction; Cardiomyopathy; Heart failure

Introduction

The heart is a dynamic organ able to self-adapt to mechanical demands, but the underlying molecular mechanisms remain poorly understood. We have previously shown that the sarcomeric Z-disk, which serves as an important anchorage site for titin and actin molecules, is not only important for mechanical force transduction but harbours as well a pivotal mechanosensitive signalosome where muscle LIM protein (MLP) and telethonin play major

roles in the perception of mechanical stimuli.[1–3] Here we focus on telethonin, a striated-muscle specific protein with a unique β–sheet structure (and no direct homologue genes), enabling it to bind in an antiparallel (2:1) sandwich complex to the titin Z1-Z2 domains, essentially "gluing" together the N-termini of two adjacent titin molecules.[4] Interestingly the telethonin – titin interaction represents the strongest protein – protein interaction observed to date.[5] Beside being phosphorylated by protein kinase D,[6] telethonin is also an *in vitro* substrate of the titin kinase, an interaction thought to be critical during myofibril growth.[7] The giant elastic protein titin extends across half the length of a sarcomere and is thought to stabilize sarcomere assembly by serving as a scaffold to which other contractile, regulatory, and structural proteins attach.[8]

Telethonin was shown to interact with MLP, hypothesized to be part of a macromolecular mechanosensor complex and to play a role in a subset of human cardiomyopathies.[2] In this context, telethonin interacts with calsarcin-1 (also known as FATZ-2 or myozenin-2, a gene recently shown to cause cardiomyopathy[9]), ankyrin repeat protein 2, small ankyrin-1 (a transmembrane protein of the sarcoplasmic reticulum)[10] and minK, a potassium channel β subunit.[11–14] In addition, telethonin was shown to interact with MDM2[15] and MuRF1[16] - E3 ubiquitin ligases with strong impact on cardiac protein turnover as well as with the proapoptotic protein Siva[17]. Recessive nonsense mutations in the telethonin gene are associated with limb-girdle muscular dystrophy type 2 G[18–20] and heterozygous missense mutations with dilated and hypertrophic forms of cardiomyopathy[1, 21–22] as well as with intestinal pseudo-obstruction.[23] Interestingly, a naturally occurring telethonin variant that has a Glu13 deletion (E13del telethonin) was initially found in patients affected by hypertrophic cardiomyopathy[21] and then later in healthy, unaffected individuals.[24–25] However, the molecular consequences of the E13del variant, especially on the telethonin-titin interaction, as well as telethonin mediated pathways in general remain unclear.

Methods

Please see also the detailed methods description in the online supplement material.

Sarcomere stretch and titin localization

Myofibrils were prepared from telethonin-deficient or wildtype tissue as described previously.[26]

In vitro protein interaction assay

Z1Z2 titin, MLP, telethonin as well as its mutants were expressed and purified as previously described.[27] Z1Z2-telethonin complexes were formed and analyzed on native gels and gel filtration columns as previously described.[4, 27]

NMR spectroscopy

U-^{2}H,^{15}N-labelled p53DBD for NMR studies was prepared using M9-medium supplemented with 1g/l $^{15}NH_4Cl$, 2g/l ^{2}H,^{13}C glucose in 99.9% D_2O (Eurisotop, Saarbrücken, Germany). Nuclear magnetic resonance (NMR) experiments were done at 293K on a Bruker Avance900 spectrometer (Bruker Biospin, Rheinstetten, Germany).

Antibodies

In the current project we employed two different anti telethonin antibodies: a mouse anti-telethonin polyclonal antibody raised against a recombinant His-tagged human full-length telethonin (western blots, immune precipitations, mouse heart and human heart sections) and a rat polyclonal anti-telethonin antibody (immunofluorescence in neonatal rat cardiac myocytes). Both the mouse and rat antibodies to human telethonin were produced by

immunizing respectively Balb/C mice or LOU/Nmir rats with purified recombinant full-length telethonin protein (1-167 aa) and their specificity checked by their ability to detect telethonin on Western blots of human heart and skeletal muscle protein. Anti-Z1Z2 titin antibody was a kind gift of Prof. S. Labeit. We used as well p21WAF1 EA10 (Calbiochem), Mdm2 2A9 and 2A10, myc 4A6 (Upstate) and actin AC15 (Abcam), anti-p53 (DO-1, FL 393, Santa Cruz), and/or mouse monoclonal p53 (1C12, Cell signalling), mouse monoclonal anti α-actinin (Sigma) and phalloidin conjugated Alexa 350 antibody. The secondary antibodies used were Alexa-labeled 633 anti-rat, Alexa- labelled 488 anti-rabbit and Alexa-labelled 488 anti-mouse (Invitrogen) antibody (please see also the Online Supplement Material for additional information).

Statistics

All animals used in the experiments were age and sex matched. All assays were analyzed in "double blind" fashion. T-tests were used to analyze differences in echocardiography (n = 8 – 9 animals per group) and for the analysis of Z-disks following sarcomere stretch. Whenever more than 2 groups were compared, ANOVA tests followed by Bonferroni's Multiple Comparison test were applied. Statistical significance was reached at $p<0.05$.

Results

To be able to perform a detailed functional analysis of cardiac performance, we generated telethonin-deficient mice by homologous recombination, replacing exons 1 and 2 with a Lac Z-neomycin cassette (Figure 1). Using this approach, telethonin was found to be transcribed as early as embryonic day 10.5 (not shown). Telethonin is a late-in protein, as such it is not a surprise that telethonin-deficient mice are born in the expected Mendelian ratios and that this protein is apparently not required during heart development.[28–29]

In contrast to recently published zebrafish and xenopus knock down models[30–31] as well as what was expected based on the available knowledge, the analysis of myocardial function by echocardiography (online table I) as well as by *in vivo* heart catheterization using 3 – 4 months old telethonin$^{-/-}$ mice under basic conditions did not reveal any abnormal parameters. Histological analysis of the spontaneous cardiac phenotype of telethonin$^{-/-}$ mice revealed no alterations, including the amount of extracellular matrix deposition (fig. 3A and B), and changes in titin-isoform composition that could be excluded based on gel electrophoresis (Online Figure I). Epifluorescence experiments showed unaltered global intracellular Ca^{2+} handling (Online Figure II) and immunohistochemistry as well as immunogold electron microscopy did not reveal any defects in telethonin-deficient Z-disks (Online Figure III).

Telethonin was shown to interact directly with the potassium channel subunit minK[13], as well as with different sodium channels such as SCN5A[23] – as a consequence we performed extensive analyses of electrocardiograms (ECG) *in vivo* as well as patch-clamp experiments *in vitro*, but did not find any significant differences in ECG parameters such as PQ interval, QRS width, QT interval, or action-potential repolarisation between control littermates and telethonin-deficient animals, without any occurrence of early or delayed after depolarisations in either group. The telethonin-minK or telethonin-SCN5A interaction may thus have little physiological relevance in the heart, at least in the mouse model (Online Figure IV). This remarkable mild cardiac phenotype despite loss of telethonin is supported by another recently published telethonin knock out mouse, where the same approach has been used to inactivate telethonin (i.e. exons 1 and 2 have been replaced by a Lac Z neomycin cassette) and where the skeletal muscle phenotype has been analyzed but almost no pathology has been detected under spontaneous conditions.[32]

Telethonin was shown to interact with the N-terminal Z1Z2 titin and as such, might have an important function in mechanically linking two titin molecules together.[4] Again, surprisingly to what we expected, stretch of single isolated myofibrils obtained from telethonin$^{-/-}$ heart or skeletal muscle did not cause any changes in Z-disk architecture or displacement of the titin N-terminus from the Z-disk, even when the sarcomeres were extended stepwise to (unphysiological) lengths of >3.2 μm to reach very high passive forces of tens of mN/mm^2 (Figure 2A, B). In contrast, compromised anchorage of the titin N-terminus was observed after removal of actin from cardiac sarcomeres (using a Ca^{2+}-independent gelsolin fragment[26]), suggesting that telethonin is mechanically relevant only when there is additional disturbance of the Z-disk (Figure 2A, B), such as impairment of the α-actinin-mediated titin-actin crosslinks.

Moreover, we reconstituted *in vitro* a complex consisting of telethonin and the N-terminal (Z1-Z2) titin domains and analyzed the effects of different human telethonin mutations on this complex formation. In contrast to several point mutations tested previously,[4] the E13del variant, which due to its presence in healthy unaffected individuals has been regarded as a polymorphism[24–25] rather than a disease causing mutation,[21] lost the ability to bind the titin N-terminus (Figure 2E–I). Consistent with previous data,[4] the deletion of this residue in telethonin leads to a loss of proper formation of the telethonin β-hairpin structure, which forms the basis for the titin binding. Given the available information on heterozygous and homozygous telethonin deficiency reported here and the fact that heterozygous loss of telethonin is not associated with any phenotype (Figure 2H), one possible conclusion is that E13del telethonin is probably a harmless naturally occurring variant unable to bind titin, hence supporting our view that telethonin, at least in mammals, performs no important structural functions. However additional effects of the E13del telethonin variant cannot be excluded and homozygous patients have not been reported.

The fulminant defects observed after actin removal in the myofibril stretch experiments led us to increase the biomechanical load under *in vivo* conditions by transverse aortic constriction (TAC). Two to three weeks after this intervention, telethonin$^{-/-}$ animals developed maladaptive cardiac hypertrophy and severe heart failure as judged by clinical signs and echocardiography (Figure 2H).

Moreover, we found an increase in focal fibrosis as well as a significant increase in TUNEL positive cells in the telethonin$^{-/-}$ animals following the TAC intervention pointing to apoptosis as a possible cause of cell death (Figure 3A–D). A detailed analysis revealed that primarily cardiac myocytes were TUNEL positive and gene expression analysis revealed differential expression of several genes involved in the apoptotic pathway(Online Figure V and VI).

Cardiac apoptosis can be efficiently induced by the tumor suppressor gene product p53,[33] a protein known to be polyubiquitinylated and marked for degradation by the E3 ubiquitin ligase MDM2.[34] Western blot analysis revealed increased p53 levels in the telethonin$^{-/-}$ animals following TAC (Figure 3E, F), whereas the apoptosis repressor with caspase recruitment domain (ARC) – another important heart specific survival factor – remained unchanged (not shown). We also found significant increases in p21 and Caspase 8 mRNA expression, both of which are p53 target genes (Figure 3 G, Online Figure VI), and a significant increase in nuclear p53 (Figure 5), supporting the finding of enhanced p53 protein levels.

Interestingly, myostatin has been implicated in the regulation of p53 and p21 expression; it is a negative regulator of cardiac growth[35–38] and is upregulated under stress.[39–41] Moreover, myostatin has also been associated with fibrosis.[42] Most importantly, telethonin

has been shown previously to interact with myostatin and to inhibit its expression.[43] Thus myostatin might be able to cause the observed effects, but we did not detect any significant changes in myostatin mRNA or protein expression (Online Figure VII and VIII).

We also performed *in vitro* experiments in neonatal rat cardiomyocytes, where we knocked down telethonin, and found a strong induction of p53 after additional doxorubicin treatment (a drug known to cause oxidative cellular stress and to induce stress responsive genes,[44] Figure 4A). In addition, we found evidence of telethonin being present in the nuclei of neonatal rat cardiomyocytes at early stages of culture (up to 2 days after plating, not shown).

Transient over-expression of telethonin in U2OS cells led to a strong down-regulation of endogenous p53 (fig. 4B, lane 2 vs. lane 4). These effects were not observed in the presence of nutlin-3, a compound preventing the interaction between p53 and MDM2, suggesting that MDM2 is required for the effects of telethonin on p53 degradation. Accordingly, expression of classical p53-responsive genes, p21 and MDM2, were suppressed due to the diminished p53 levels. This prompted us to investigate the underlying molecular mechanism in more detail and we found direct interaction of telethonin and MDM2 by co-immunoprecipitation assays (Online Figure IX), supporting earlier observations by Tian and co-workers.[15] In addition, a direct interaction of p53 and telethonin was detectable by co-immunoprecipitation experiments (Figure 4C) as well as pull down assays (Figure 4D). Native and SDS-PAGE gel electrophoresis indicated the formation of a high-molecular weight complex (~ 150 kDa) between full-length telethonin (wild type and E13del) and full length p53 *in vitro* (Figure 4D, right panels).

Static light scattering and NMR analysis additionally showed that the interaction involves the p53 DNA binding domain (p53DBD). Static light scattering indicated a molecular weight of 163 kDa for this complex suggesting that it might consist of multiple telethonin and p53DBD molecules (Figure 4E). NMR spectroscopy further confirms that the telethonin interaction involves the p53DBD (Figure 4F). NMR chemical shift differences between the free and telethonin-bound p53DBD reveal that telethonin contacts the β-sheet of p53DBD, at a site that is remote from the DNA binding interface (Figure 4F, Online Figure X). Fluorescence polarization (FP) experiments (Figure 4G) and Surface Plasmon Resonance (SPR, Biacore) experiments (Online Figure X) show that the interaction between telethonin and p53DBD has a low micromolar dissociation constant (K_D = 2.2 ± 0.2 μM and 0.765 ± 0.03 μM for FP and SPR, respectively, Online Figure X). These values are comparable to other protein-protein interactions that have been mapped to p53DBD.[45–47] It is interesting to note that the interaction of telethonin with titin (Figure 2G) also preferentially involves the β-sheets of the titin Z1-Z2 domains forming intermolecular β-strand contacts. Similar interactions might contribute to the stabilization of the telethonin-p53DBD complex.

We performed as well a series of F-actin, α-actinin, telethonin and p53 co-localization studies and found that p53, in contrast to the Z-disk localization of telethonin, is not clearly detectable under spontaneous conditions, neither in the *in vivo* setting nor in isolated neonatal rat cardiomyocytes in *vitro* (Online Figure XI, XII). However, after biomechanical or oxidative stress *in vivo,* such as TAC (Figure 5), or doxorubicin treatment *in vitro* and *in vivo* (Online Figure XIII, XIV), we observed a strong induction of p53 in cardiomyocyte nuclei, which is well in accordance with previously published data on p53.[48–49] Moreover, under both stress conditions telethonin co-localized with p53 in cardiomyocyte nuclei (Online Figure XIII, XIV). However, an even stronger increase in p53 nuclear expression was observed after TAC in telethonin deficient animals, supporting the results of our previous Western blot analysis (Figure 3 and Figure 5). Telethonin/p53 colocalization was also observed when we transfected neonatal rat cardiomyocytes in *vitro* using a GFP-telethonin construct (Online Figure XV).

Based on these data we assumed that telethonin at least supports MDM2 mediated p53 degradation. As a consequence we aimed to analyze the effects of telethonin overexpression on myocardial function under *in vivo* conditions and generated telethonin transgenic animals. We used the myocardium specific alpha myosin heavy chain promoter and a FLAG-tagged mouse telethonin cDNA (Figure 6). Again, to our surprise these animals did not exhibit any spontaneous phenotype (online table II).[50] Of note, they develop less apoptosis as well as less p53 expression in comparison to wildtype littermate controls after TAC (Figure 6). Particularly the decrease in apoptotic (TUNEL positive) cells in telethonin transgenic animals is interesting and might indicate potential protective effects of telethonin overexpression.

We then assumed that p53 determines the negative outcome in telethonin deficient animals following biomechanical stress and we used a transgenic line overexpressing a well characterized dominant negative p53 mutant (i. e. the Arg193Pro mutation)[51–52] to inactivate this protein in the telethonin$^{-/-}$background (Figure 7 A). dnp53/telethonin$^{-/-}$ double transgenic animals did not develop any spontaneous phenotype and they did not exhibit any significant change in myocardial function following TAC. However, genetic inhibition of p53 in the telethonin deficient background significantly inhibited the increase in apoptosis found after biomechanical stress in telethonin$^{-/-}$animals alone (Figure 7 B).

In order to study telethonin mRNA expression in the human heart we analyzed myocardial samples from end-stage heart failure patients and found significant down-regulation compared to normal donor hearts (Figure 7 C), as well as an increase in nuclear telethonin (Figure 7D). This may have implications for p53 expression and p53-related apoptosis both of which have previously been shown to be elevated in these patients. We also found down-regulation of telethonin in acute donor organ failure suggesting this effect is not restricted to the setting of chronic end-stage failure.

Discussion

Here we demonstrate a model where a primary defect in an integral Z-disk component is not associated with any cardiac phenotype or functional abnormality under basal conditions.[53] However, pressure overload causes a maladaptive response in homozygous telethonin$^{-/-}$ hearts, ultimately leading to global heart failure *in vivo*. Loss of the p53-ligand telethonin is associated with an increase in p53 as well as elevated apoptosis following an increase in afterload – which is the first description of a Z-disk component to do so. Moreover, by binding to p53's DNA binding domain, telethonin is potentially able to repress the function of this important transcription factor.

Telethonin, which was shown to be phosphorylated *in vitro* by the titin kinase,[7] does not seem to have a function during embryonic development *in vivo*. A recent study[54] reported a defect in C2C12 myoblast differentiation when telethonin was downregulated by the use of siRNAs. It remains to be elucidated whether there are differences *in vivo* and *in vitro* or whether the telethonin siRNAs per se exhibit off target effects that account for the observed differences. In addition loss of telethonin in zebrafish or xenopus is associated with a spontaneous defect[30–31] as such it will be important to elucidate in future whether telethonin in mammalian hearts acquired additional functions during evolution or if so far unknown telethonin homologue genes are upregulated or if differences in Z-disk structure account for the observed differences. Our data are generally consistent with a recent report, whereby telethonin binds in an antiparallel (2:1) sandwich complex to the titin Z1-Z2 domains.[4] However, telethonin clearly is not required to stabilize the sarcomere structure. Instead, telethonin may serve as a pivotal element in cardiac signalling by controlling apoptosis and cell death via p53. Our data are compatible with a direct molecular link between the

sarcomeric Z-disk, cardiac performance, as well as gene transcription and cell survival ("mechano-transcriptional coupling - MTC"), although additional data need to be provided to entirely support such a functional link. It might well be that Z-disk proteins carry at least two different functions: 1) a structural function which might be dismissible particularly in "peripheral" Z-disk proteins and 2) a regulatory function which, as in this case, might be much more important. One implication of this could be that cardiomyopathy and associated heart failure, which can be caused by mutations in Z-disk components (now regarded as a "hot spot" for these mutations[55]) might be seen as a disease caused by "defects in cardiac regulation" or of "defects in mechano-transcriptional coupling".

In conclusion, this study might change the previous concept on Z-disk structure, which we now suggest to also be a pivotal node for apoptosis – essentially by linking telethonin to p53 (Figure 8). In contrast to previous views, telethonin is not an indispensable component of the cardiac titin anchoring system and cardiac specific telethonin overexpression is not immediately associated with Z-disk pathology and as such is compatible with life. Instead, under normal conditions, actin crosslinking may be sufficient to keep the sarcomere structure viable, despite loss of telethonin. With an increase in hemodynamic load or an increase in biomechanical or oxidative stress, however, telethonin deficiency leads directly to enhanced p53 levels and as such promotes an increase in apoptosis and cell death, thus initiating the development of heart failure, an effect which might be called "mechanoptosis".

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

dn	dominant negative
SERCA	sarcoplasmic reticulum ATPase
BNP	brain natriuretic peptide
ANF	atrial natriuretic factor
α-MHC	alpha myosin heavy chain
β-MHC	beta myosin heavy chain
NLS	nuclear localization sequence
P53DBD	p53 DNA binding domain

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Novelty and Significance

What is known?

- Telethonin is a small (19 kDa) muscle-specific protein
- Telethonin is localized to the sarcomeric Z-disk where it interacts with the giant protein titin.
- Telethonin mutations are associated with various diseases such as limb girdle muscular dystrophy 2 G (LGMD 2G), cardiomyopathy and intestinal pseudoobstruction

What new information does this article contribute?

- Telethonin deficiency is not associated with a spontaneous phenotype, at least not in the mammalian heart
- Telethonin is not essential for the mechanical stability of the Z-disk
- Telethonin promotes cardiac myocyte survival by suppressing p53 mediated apoptosis

Telethonin mutations are associated with several diseases but the underlying molecular mechanisms remain not well understood. To analyze the in vivo function of telethonin we generated genetically altered mouse models and found that telethonin is a dispensable component of the sarcomeric Z-disk. Deletion or cardiac-specific overexpression of telethonin was not associated with a spontaneous cardiac phenotype. However, our results showed that telethonin modulates the turnover of the pro-apoptotic protein p53 after biomechanical stress. This novel finding links telethonin directly to apoptosis (“mechanoptosis”), which is considered a new cell death associated pathway. We also observed a reduction in the eexpression of telethonin and an increase in its nuclear abundance in myocardial samples from end-stage heart failure patients, indicating that changes in telethoninmay contribute to cardiac maladaptation. These findings suggest that telethonin, together with other Z-disk associated proteins, might have novel functions in anti-apoptotic cell survival pathways.

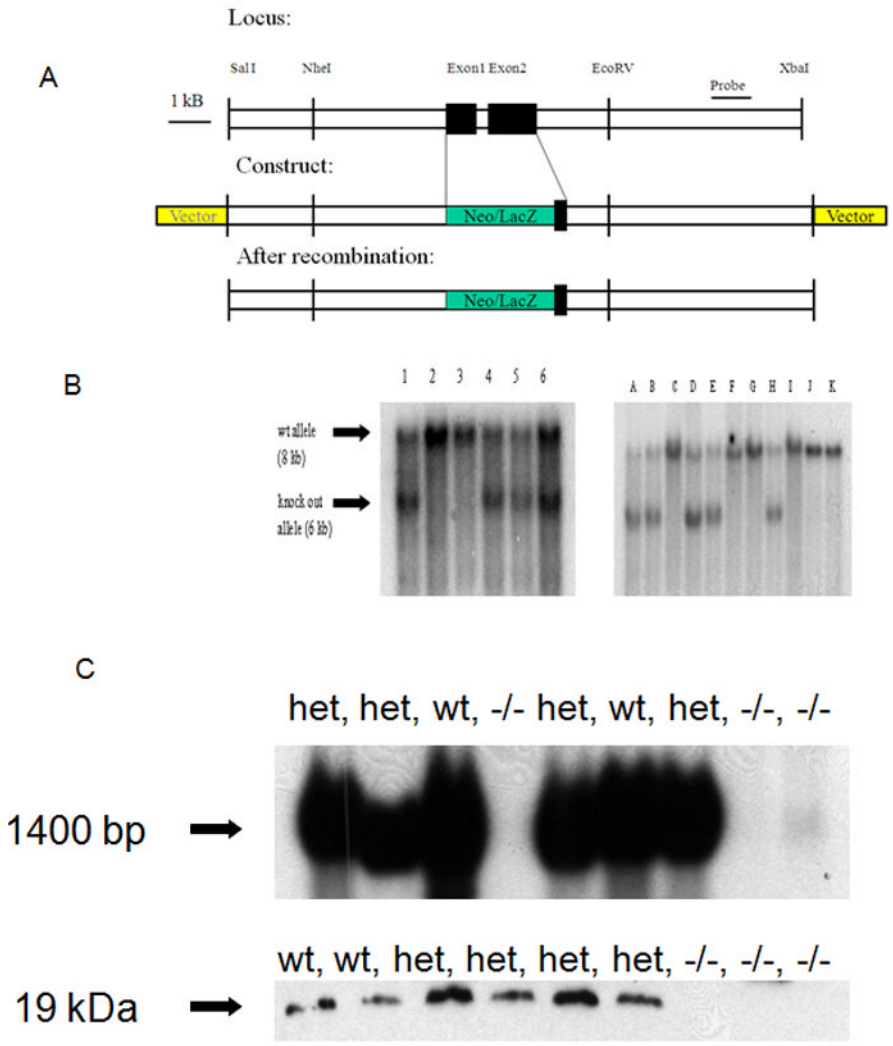
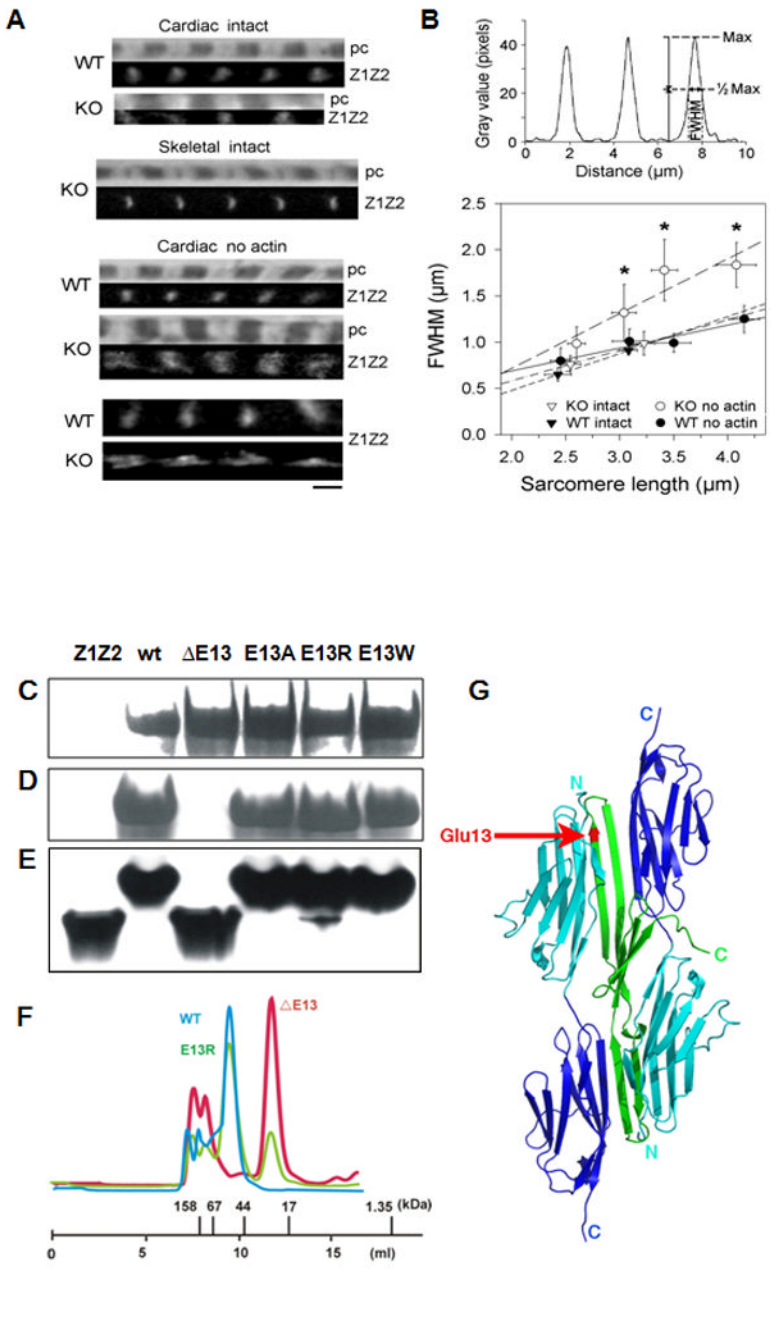


Figure 1. Generation of telethonin$^{-/-}$ animals

a) General strategy for gene targeting. The gene for telethonin is encoded by two exons, restriction sites are indicated. The gene was replaced by a LacZ/Neomycin cassette (targeting construct is indicated)

b) Southern hybridization of embryonic stem cells (left panel: different stem cell lines marked 1–6) as well as of resulting animals (right panel: different animals marked A–K)

c) Telethonin mRNA expression, analyzed by northern blot (upper row), as well as protein expression, analyzed by Western blotting, indicates that telethonin$^{-/-}$ results in a “true null allele”.



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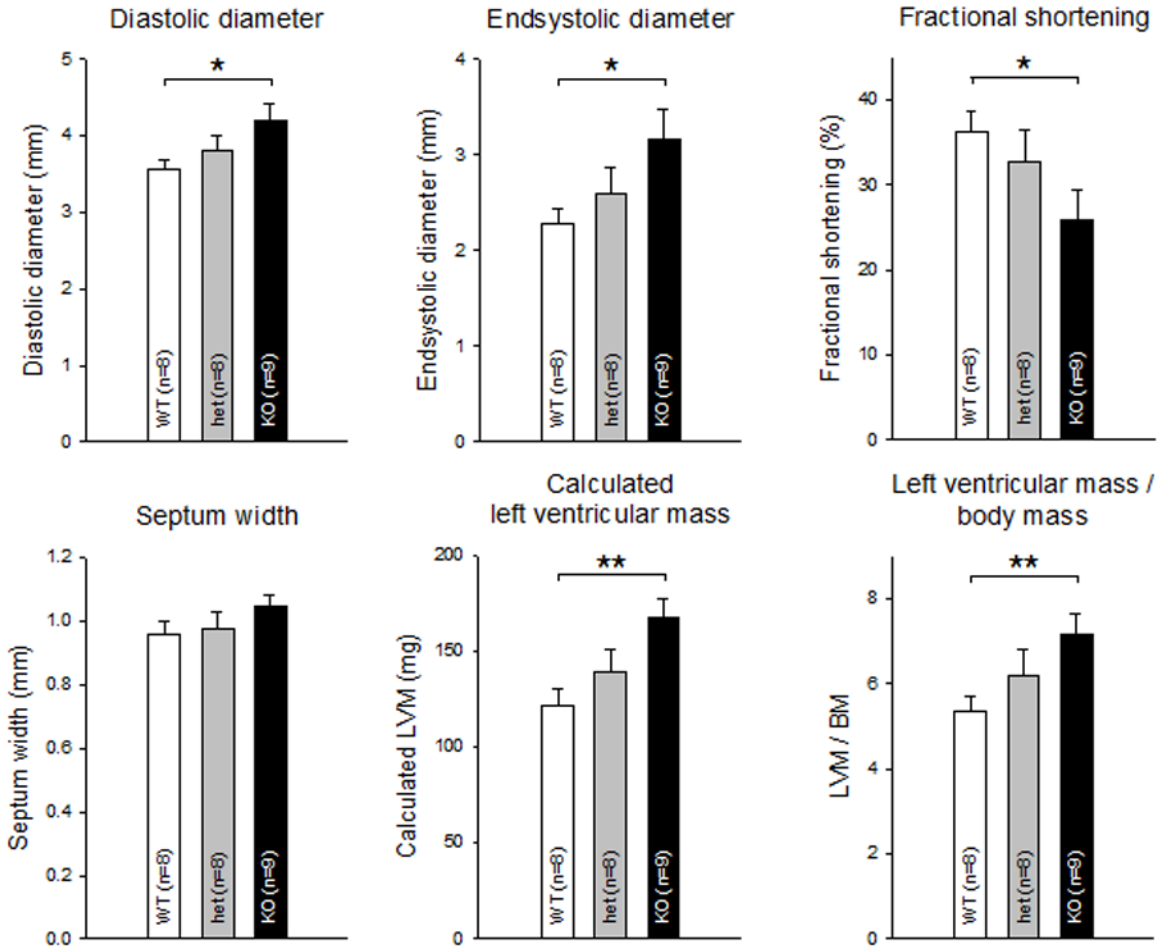
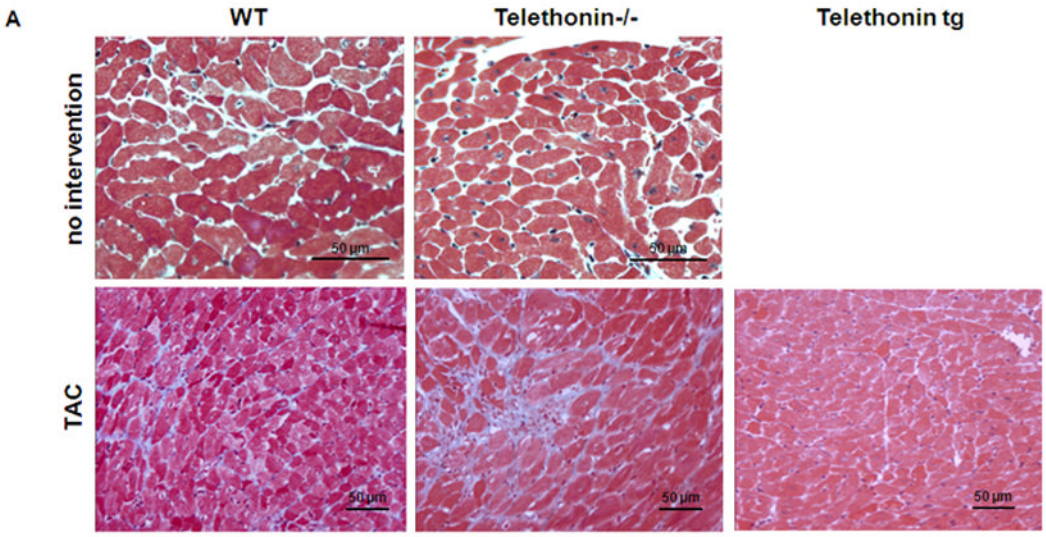
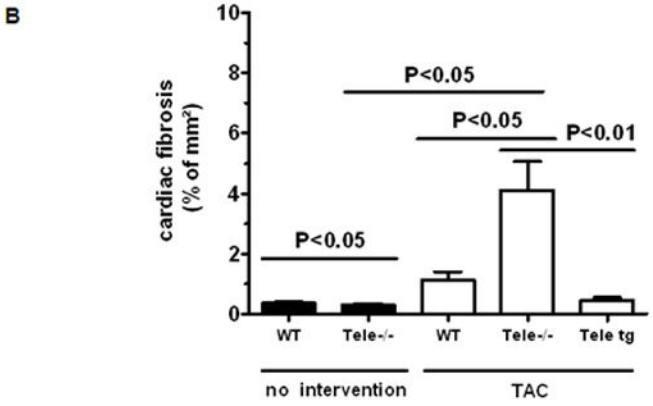


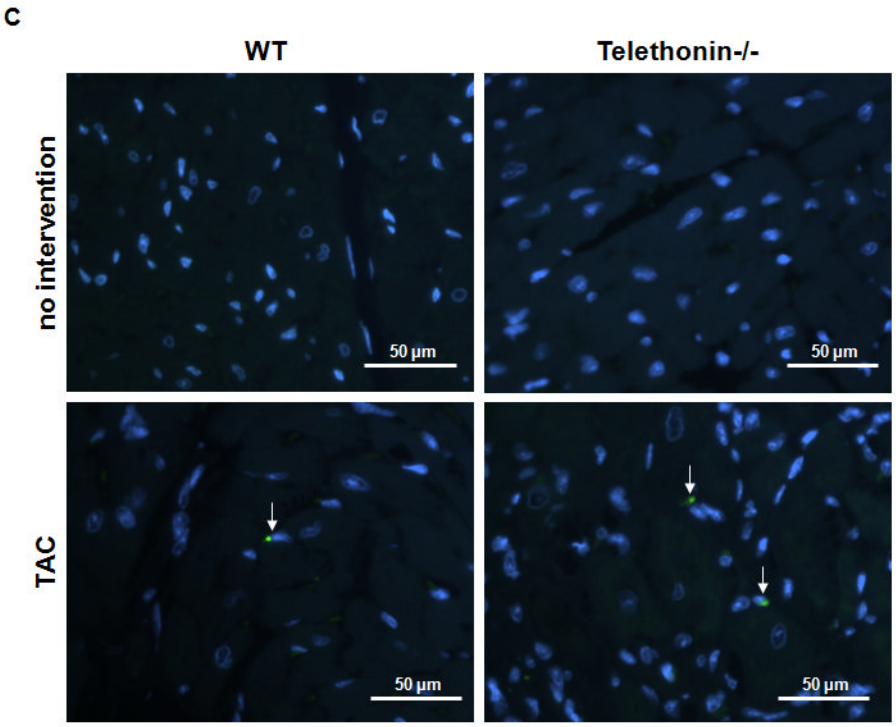
Figure 2. Probing functional consequences of telethonin deficiency at the subcellular and organismic levels

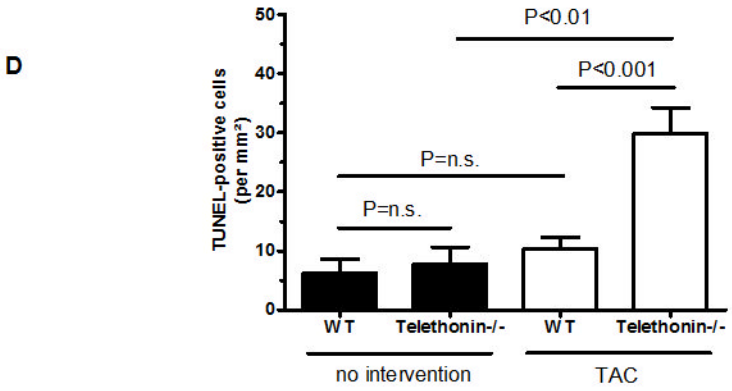
A, B) Myofibrils were isolated from either wildtype (WT) or homozygous telethonin-deficient (KO) mouse hearts and stretched to a desired sarcomere length (SL) under non-activating conditions. Then, myofibrils were stained with an antibody to the telethonin-binding titin-domains, Z1/Z2, and secondary labelled using FITC-conjugated IgG. (A) Phase-contrast (pc) and immunofluorescence (Z1Z2) images of stretched myofibrils from cardiac muscle before actin extraction; telethonin-deficient skeletal muscle; and cardiac muscle after actin extraction using a Ca^{2+}-independent gelsolin fragment (shown are myofibrils at two different stretch states). Scale bar, 2 μm. (B) (*top*) Quantitation of the broadness of the titin-label in the Z-disk by determining the full-width at half-maximum (FWHM) peak height on intensity profiles along the myofibril axis. (*bottom*) Average widths of Z1Z2-titin label in WT and KO cardiac myofibrils at different SLs, before and after actin extraction. Data are means±SD (n=3–6), * P<0.05 in Student's t-test.

C) Pull-down with MLP. N-terminus of titin (Z1Z2, used as a control), Tel (1-90), Tel (1-90, dE13), Tel (1-90, E13A), Tel (1-90, E13R) and Tel (1-90, E13W) were incubated with a recombinant GST-MLP fusion protein and pulled down with glutathione-sepharose 4B beads (anti-GST antibody anti-rabbit, Pharmacia Biotech, USA).
D) Pull-down with Z1Z2. Same experiment as in C), except that instead of MLP an H-tagged N-terminus of titin Z1Z2 was used (Pull down with Ni^{2+}-NTA beads, QIAGEN, Germany, blot with antibody against telethonin).
E) Native PAGE analysis of titin/telethonin complexes formed from telethonin and its mutants with Z1Z2. On the native gel, only the Z1Z2-telethonin complex and Z1Z2 were visible.
F) Analysis of the Z1Z2-telethonin complex formation by size exclusion chromatography in combination with static light scattering. The complexes were loaded onto a sephadex-column, molecular masses were calculated to be 23.0 (Z1Z2) and 55.4 (Z1Z2-telethonin complex) kDa.
G) Structure of the telethonin-titin Z1-Z2 complex, the arrow indicates glutamate 13 (E13), important for stabilizing the β-hairpin structure.
H) Functional analysis of telethonin deficiency *in vivo*: telethonin$^{-/-}$ animals developed 2 – 3 weeks after transverse aortic constriction (TAC) a defect in myocardial function (increased endsystolic and enddiastolic diameters, decrease in fractional shortening as well as increased left ventricular mass (LVM) and LVM per body mass (* $p<0.05$, ** $p<0.01$, error bars indicate standard error of the mean (SEM)).









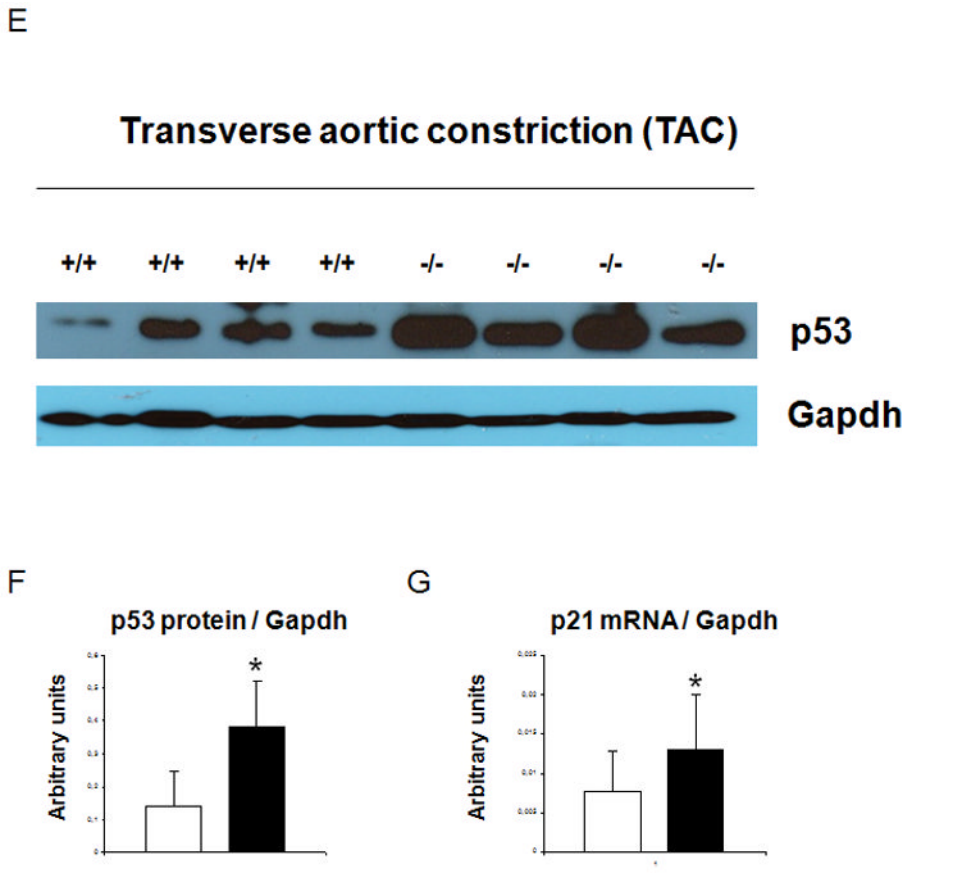


Figure 3. Telethonin – analysis of fibrosis, apoptosis and p53

A) Wildtype (WT) and telethonin knockout (KO) hearts were analyzed for the presence of fibrosis (masson trichrome stain) without intervention and after transverse aortic constriction (TAC). Telethonin transgenic animals did not develop any significant increase in fibrosis.

B) Quantification of fibrosis, please note the significant increase in fibrosis in the telethonin deficient animals (without intervention (black bars) and after TAC (white bars); error bars indicate standard deviation (SD)).

C) Wildtype (WT) and telethonin knockout (KO) hearts were analyzed for the presence of apoptosis without intervention and after transverse aortic constriction (TAC).

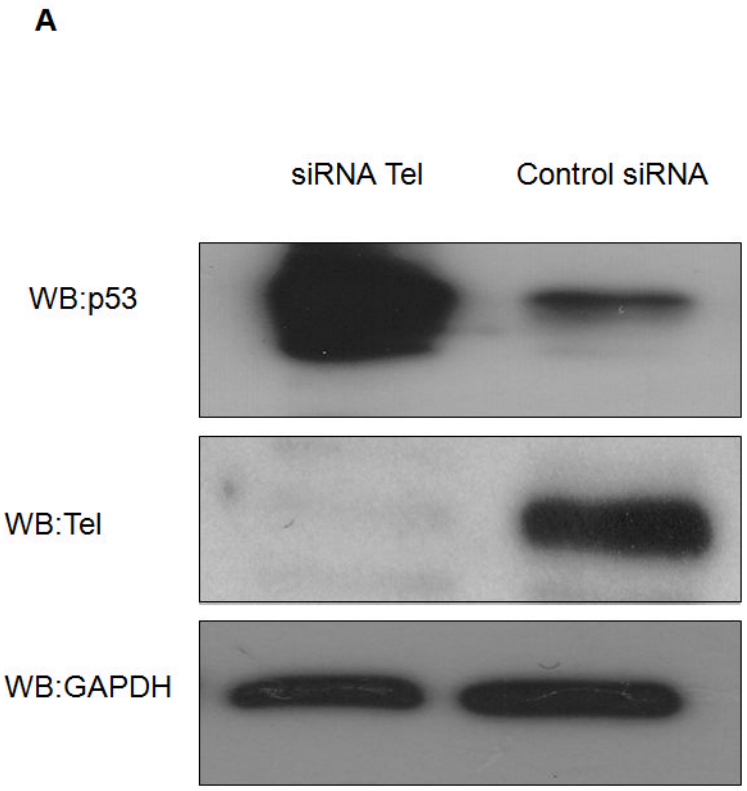
D) Quantification of apoptosis, please note the significant increase in apoptosis in the telethonin deficient animals (without intervention (black bars) and after TAC, white bars; error bars indicate standard deviation (SD)).

E) Western blot analysis of p53 expression in hearts of telethonin knockout as well as corresponding wildtype litter mate control hearts. Equal loading of the membrane has been confirmed by GAPDH gene expression. Please note the significant increase in p53 expression in the telethonin$^{-/-}$ animals.

F) Quantification of p53 protein expression. Data have been normalized to GAPDH. Please note the significant increase in p53 expression in the telethonin$^{-/-}$ animals (n=4 animals per group, white bars: wildtype animals, black bars: telethonin$^{-/-}$ animals, * = $p<0.05$; error bars indicate standard deviation (SD)).

G) Relative levels of p21 mRNA transcripts in left ventricles. We used hearts obtained from WT and telethonin$^{-/-}$ mice subjected to TAC for 3 weeks and analyzed mRNA expression by quantitative real-time PCR. Please note the significant increase in p21 gene expression,

which is a p53 target gene. (white bars: wildtype animals (n = 5), black bars: telethonin$^{-/-}$ animals (n = 12), *p<0.05 against WT-TAC, error bars indicate standard deviation (SD)).



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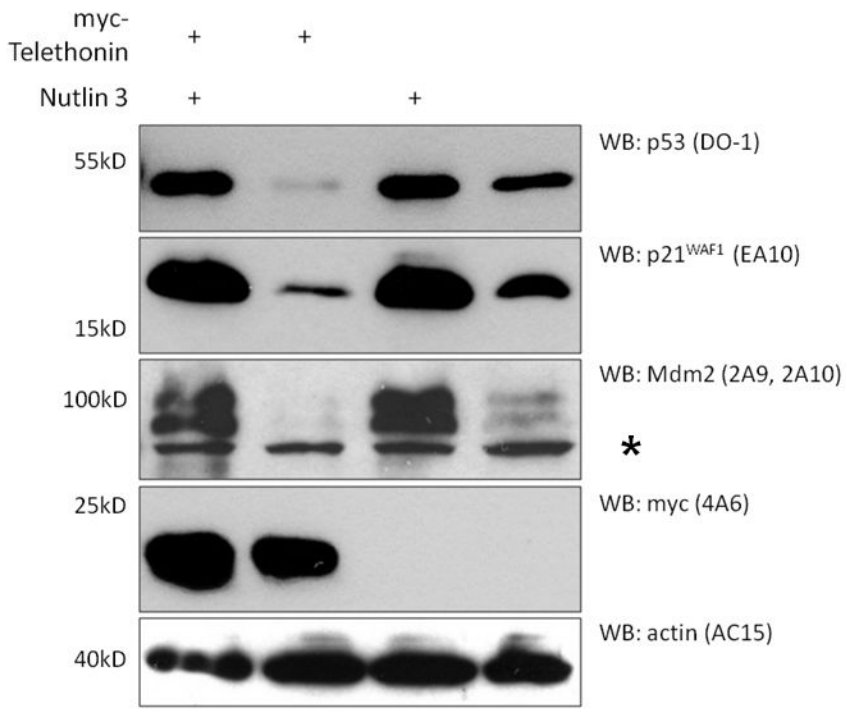

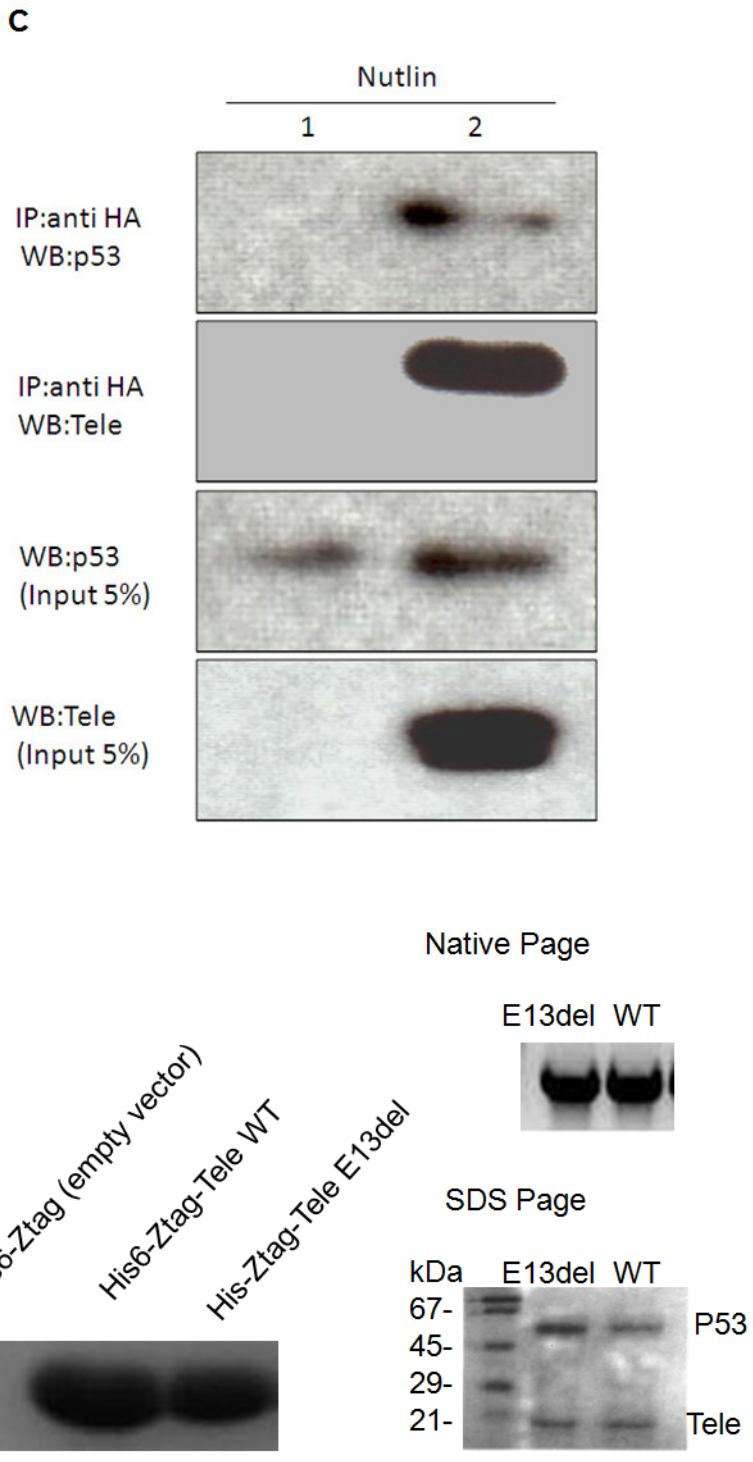

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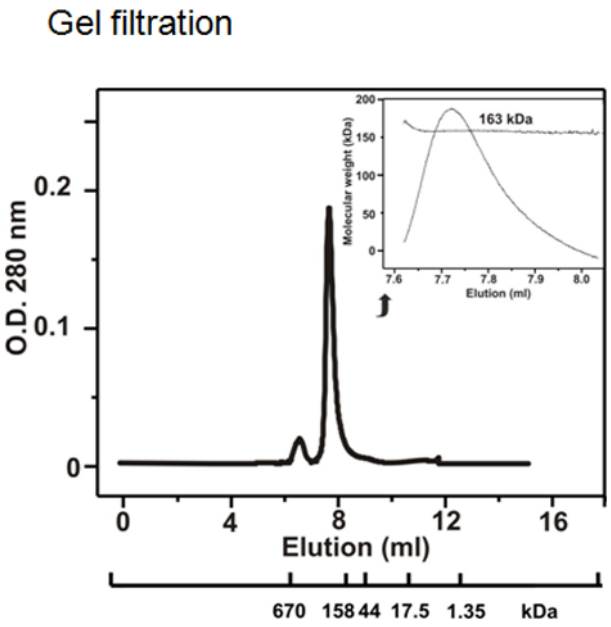


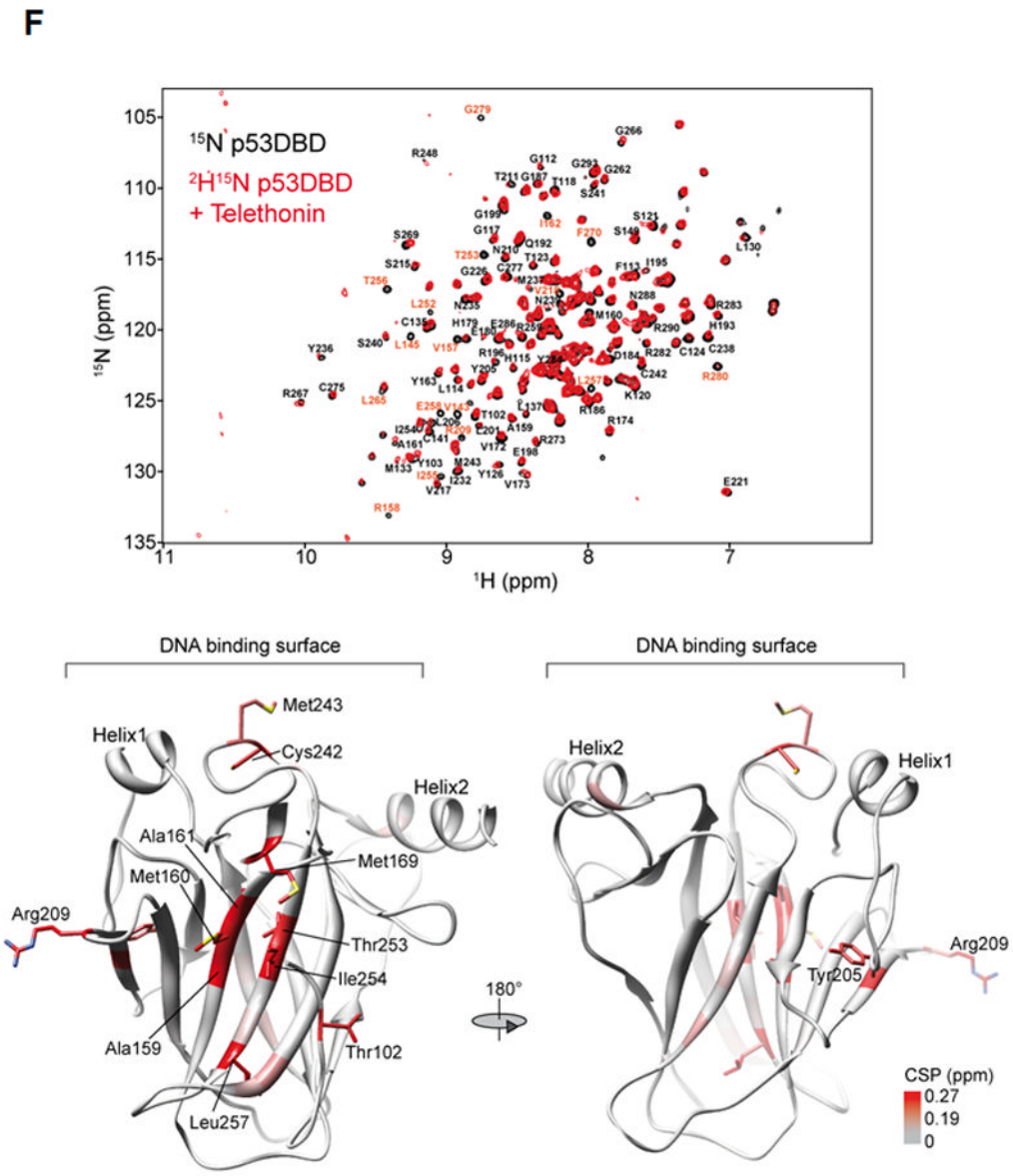


Figure 4. Telethonin/p53 interaction

A) After 48 h of silencing with siRNA telethonin, neonatal rat cardiomyocytes were incubated with doxorubicin (1μM) for the next 18 h. The cells were harvested and whole cell extracts were used for Western blot analysis. Membranes were probed with mouse polyclonal telethonin as well as with rabbit polyclonal p53 antibodies (Santa Cruz). GAPDH was used as a loading control. Please note the strong induction of p53 when telethonin is knocked down.

B) U2OS cells were transfected with myc-telethonin and treated with nutlin-3. Cells were harvested 36 hours post transfection in RIPA buffer. Cell lysates were used for Western blot analysis. * unspecific signal

C) Interaction between telethonin and p53.

This interaction was detected in U2OS cells between endogenous p53 and transfected HA-tagged telethonin in the presence of Nutlin (8μM). The IP samples were subjected to SDS-PAGE, blotted and the blot probed with anti-p53 monoclonal antibody (DO-1, Santa Cruz).

Lane 1: U2OS cells transfected with empty plasmid, IP was performed with anti-HA antibody; Lane 2: telethonin transfected U2OS cells, IP was performed with anti-HA antibody

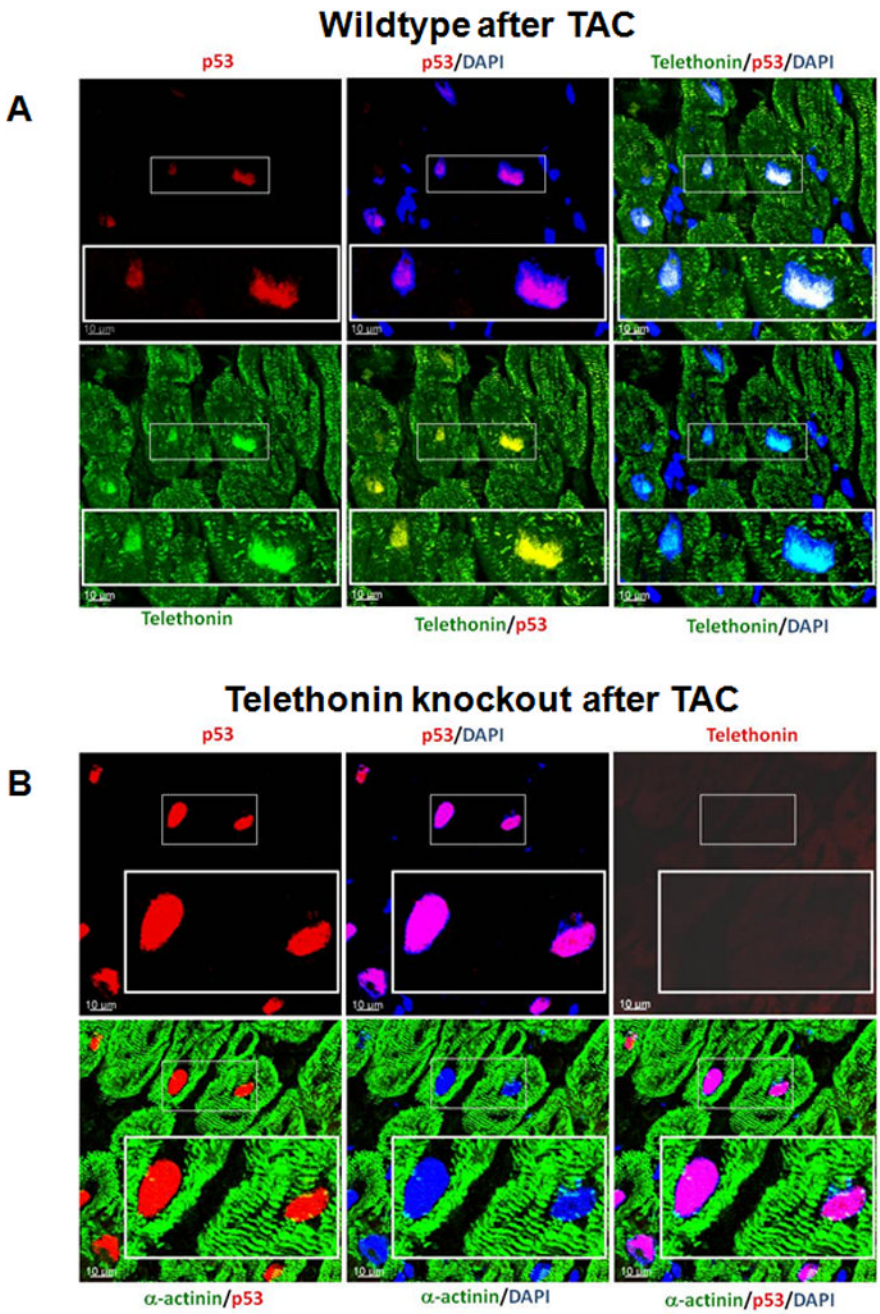
E) Telethonin wild type or E13del mutant form a complex with the DNA binding domain of p53 (p53DBD). The complex was separated using size-exclusion chromatography (Superdex 200) and the molecular weight was measured by static light scattering.

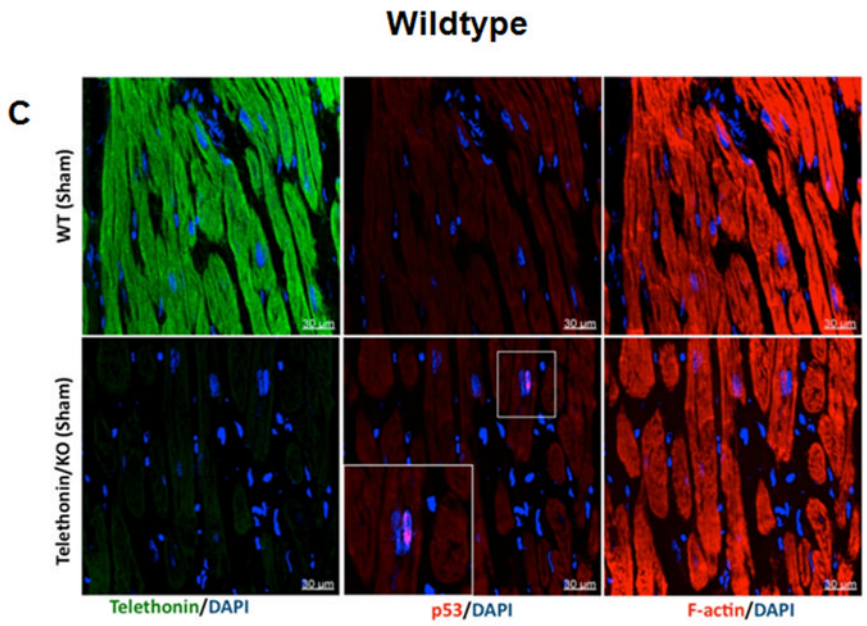
F) Interaction between p53 DNA binding domain (DBD) and telethonin.

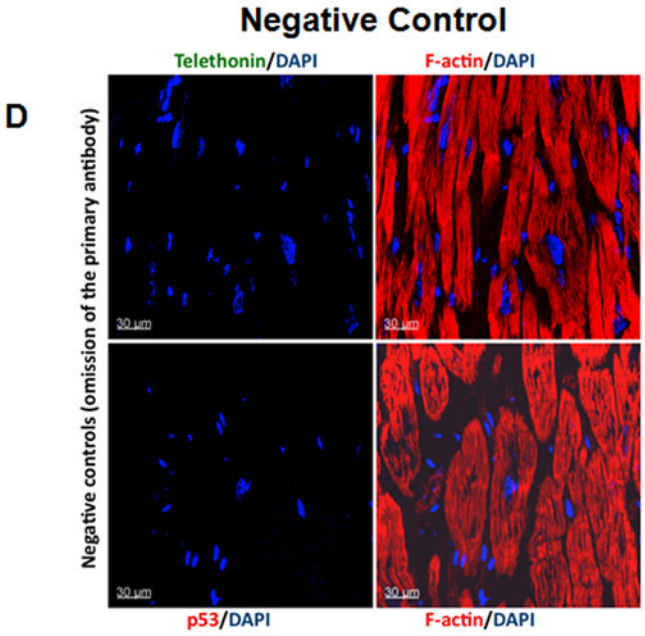
Top panel: Superposition of $^{1}H,^{15}N$ TROSY experiments of 50 μM ^{15}N-labelled p53DBD (black) and the complex of $^{2}H,^{15}N$ p53DBD and telethonin (red) recorded at 900 MHz proton frequency. Some signals within p53DBD experience substantial line-broadening in the complex (red labels). The signals of many other amino acid residues show chemical shift perturbations upon complex formation (black labels).

Bottom panel: CSP values mapped onto the structure of p53DBD. Significantly affected residues are labelled and cluster to one side of the β-barrel region of p53DBD. The p53 DNA binding region consisting of helix 1 and 2 (H1, H2) and loop 3 (L3) is indicated. Colour coding: yellow to red, above mean value plus one standard deviation; red, above mean value plus two standard deviations (see also middle panel).

G) Fluorescence polarization of 0.5 μM fluorescein-labelled p53DBD upon the addition of telethonin in 10 mM sodium phosphate pH7.2, 1 mM TCEP. Fitting to an apparent one-site binding model (red line) yields a dissociation constant of 2.2 ± 0.2 μM. Three individual measurements were performed for error estimation.







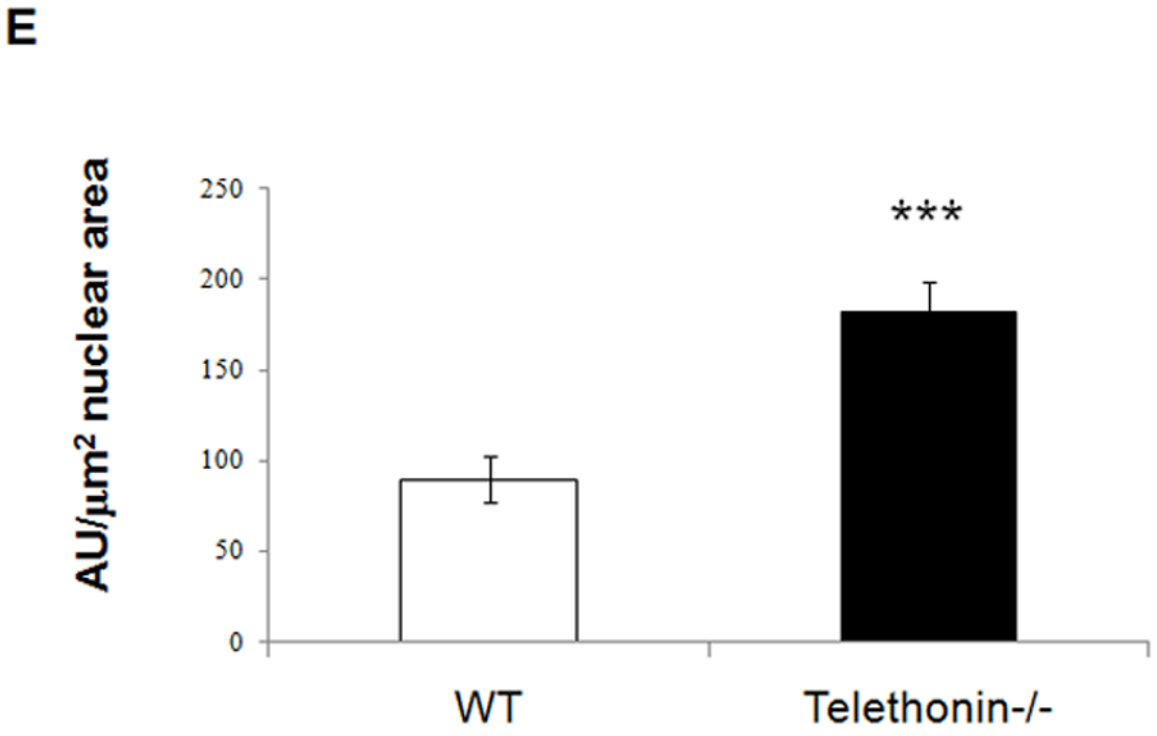


Figure 5. Analysis of telethonin/p53 co-localization

A–D) Representative confocal micrographs showing nuclear localization of p53 in wildtype (WT) (A) and telethonin knockout hearts (B) after TAC. Telethonin and p53 localization has also been analyzed under spontaneous conditions (C), the control panel is provided under D. Please note: telethonin is not dectectable in telethonin deficient hearts and in telethonin deficient animals just a very few nuclei having an apparent tendency to be positive for p53 were observed (boxed region). However, this phenomenon was so rare that it does not differ substantially from WT sham operated mice. Note as well that in wildtype mice telethonin is present in the nuclei after TAC where it colocalizes with p53. Inserts are higher magnifications of the boxed regions.

(p53 in red, DAPI (nuclear staining) in blue, telethonin or α-actinin in green mark cardiomyocytes)

E) It is also evident that p53 nuclear expression levels are much higher in telethonin knockout mice in comparison to wildtype mice after TAC (graph, *** $p<0.005$; error bars indicate standard deviation (SD)).

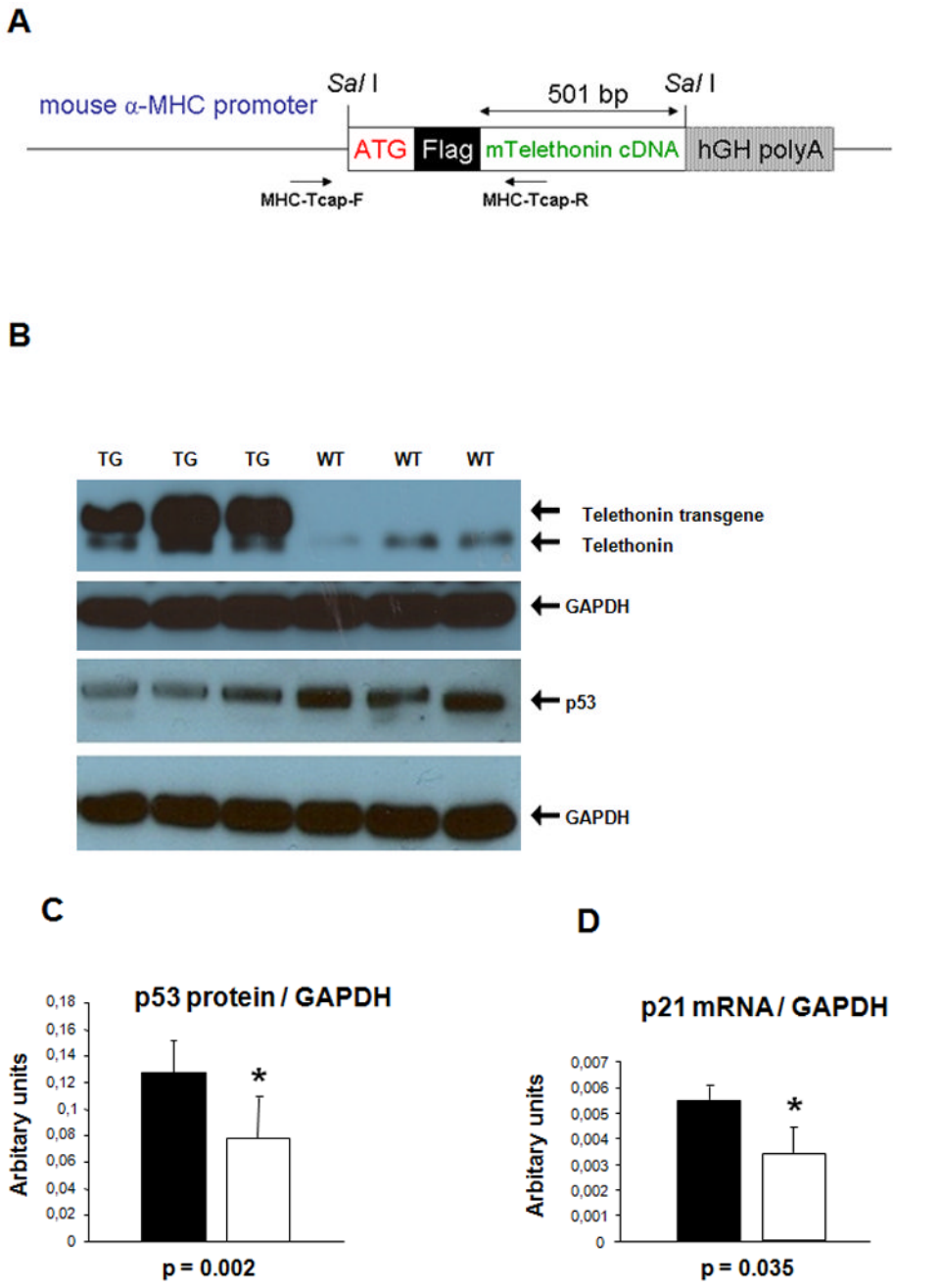
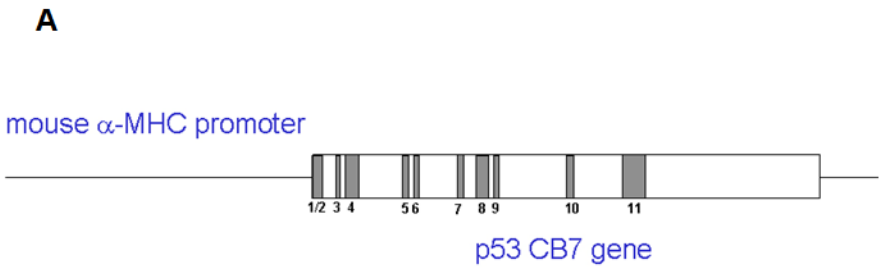


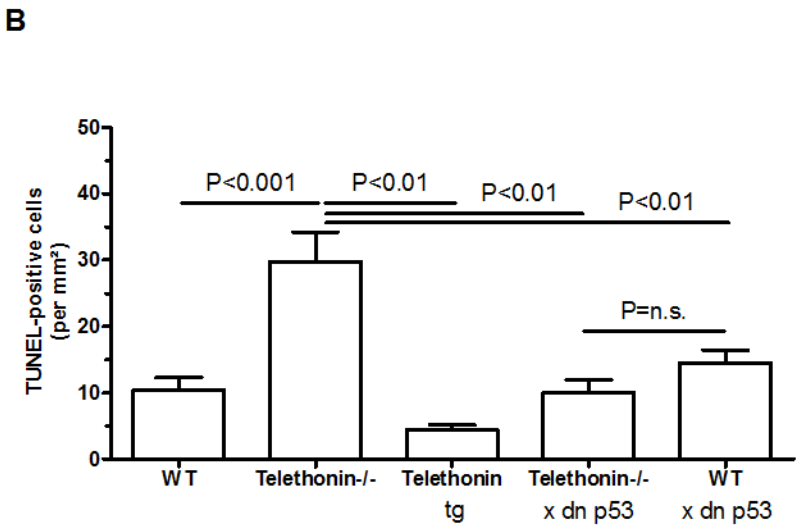
Figure 6. Analysis of the effect of telethonin on p53 *in vivo*

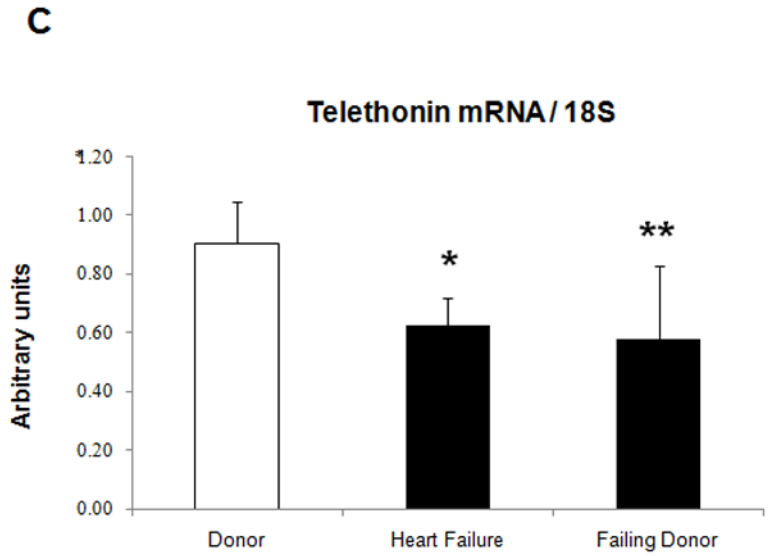
A) Schematic diagram of the construct used to generate telethonin transgenic animals. An alpha myosin heavy chain promoter was used for myocardium specific expression of a FLAG-tagged mouse telethonin cDNA. Human growth hormone poly A tail has been used to enhance stability of the transcript. MHC-telethonin (Tcap)-F and R primers were used for genotyping.

B) Western blot analysis of telethonin and p53 gene expression in telethonin transgenic (TG) versus wildtype littermate control animals (non transgenic, NTG) 2 weeks after transverse aortic constriction (TAC). Please note the decrease in p53 gene expression in telethonin transgenic animals.

C) Quantification of p53 protein levels in telethonin transgenic animals. Data are normalized to GAPDH. Please note: telethonin overexpression decreases p53 protein levels. (White bars: wildtype animals (n = 3), black bars: telethonin transgenic animals (n = 3), * = $p<0.05$; error bars indicate standard deviation (SD).
D) Quantification of p21 mRNA expression in telethonin transgenic animals. p21 is an important target gene of p53 and the decrease in p21 mRNA is well in accordance with loss of p53 protein expression. (White bars: wildtype animals (n = 3), black bars: telethonin transgenic animals (n = 3), * = $p<0.05$, ** = $p<0.01$; error bars indicate standard deviation (SD).







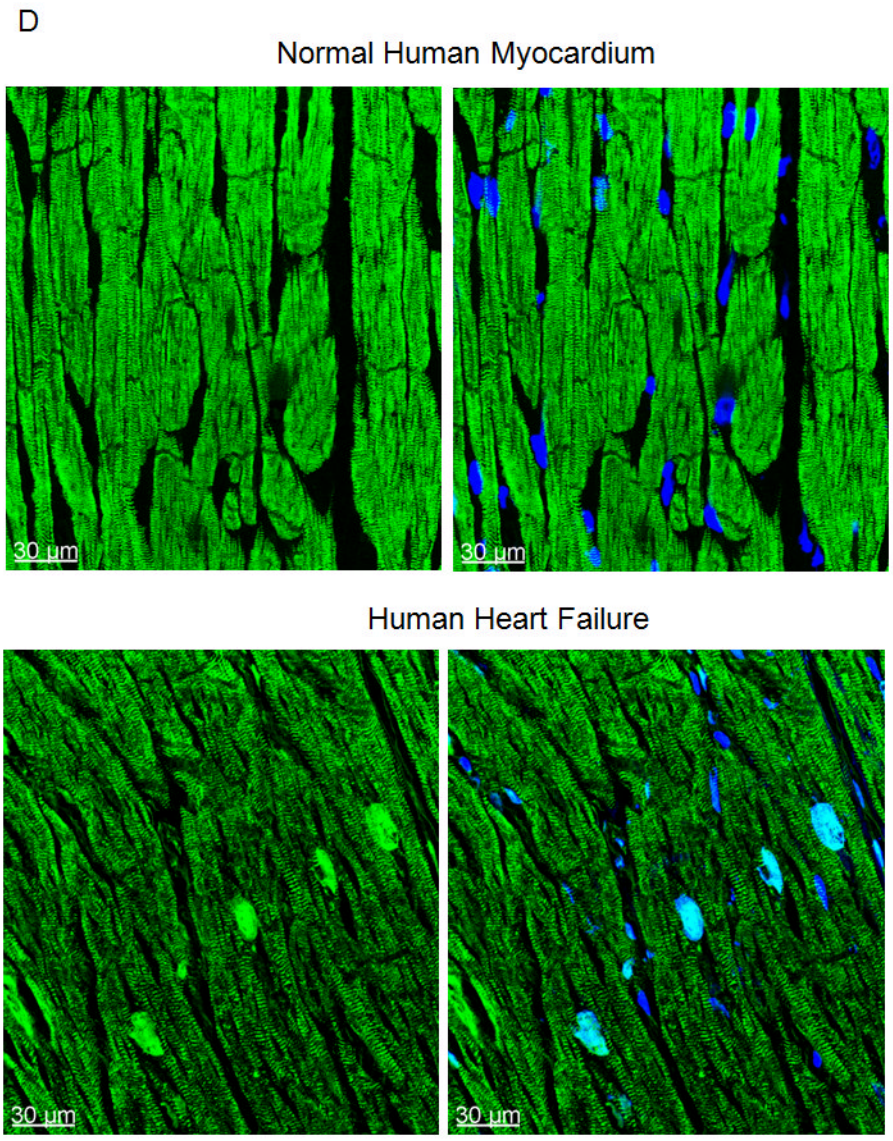


Figure 7. Inhibition of p53 and its effect on telethonin deficiency after TAC and implications for human disease

A) Schematic diagram of the CB7 construct, which encodes the Arg193Pro mutation, a well known dominant interfering phenotype (i. e. dominant negative (dn) p53) [51]. These mice have been crossed into the telethonin knockout background resulting in dn p53/telethonin double transgenic animals. The numbers refer to p53 exons (grey boxes); 1/2 indicates that a fusion was made between exons 1 and 2.

B) Quantification of apoptosis after transverse aortic constriction (TAC). Please note that p53 inhibition in the telethonin knockout background prevents the increase in apoptosis. (Wildtype (n=15), telethonin−/− (n=11), telethonin transgenic (n=4), dn p53 (n=13) and dn p53/telethonin double transgenic animals (n=7); error bars indicate standard deviation (SD)).

C) Quantification of telethonin mRNA by quantitative real time PCR in ventricular myocardium from normal donor organs (n=8), end-stage heart failure patients taken at the time of transplantation (heart failure n=7) and from failing donor organs with EF<30% (n = 9). Data are shown as mean ± standard deviation (SD). * = $p<0.05$, ** = $p<0.01$ versus donors.

D) Analysis of telethonin localization in human hearts. Please note: under physiological (no disease) conditions telethonin is present at the sarcomeric Z-disk but not in cardiac nuclei (upper panel). In heart failure telethonin is less present at the sarcomeric Z-disk and more present in cardiac nuclei (lower panel).

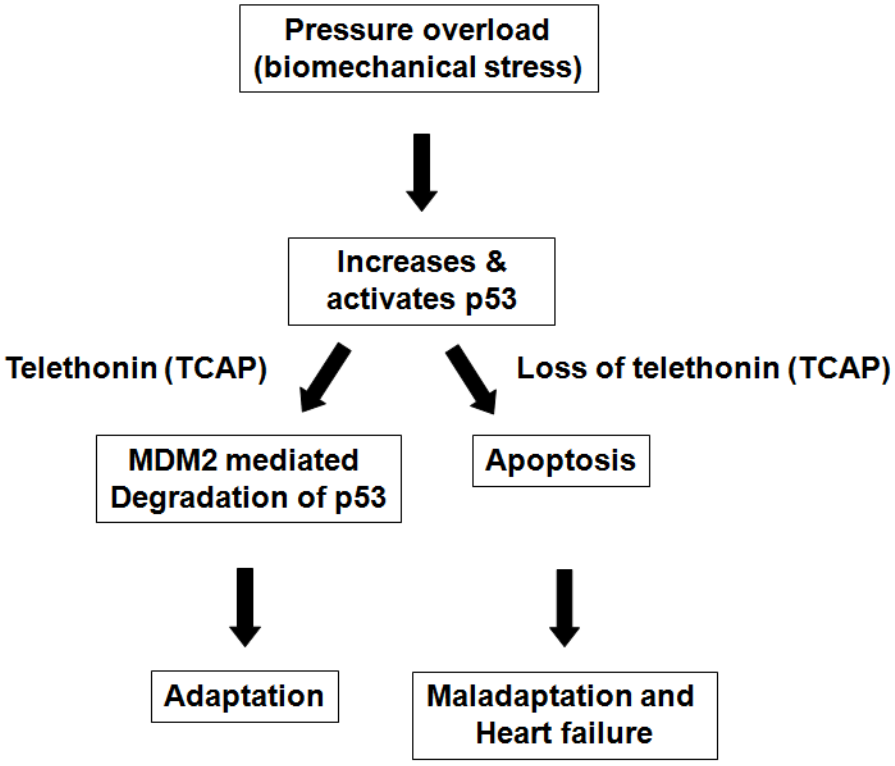


Figure 8. Proposed model depicting telethonin as an essential signalling component in the heart

Based on our *in vitro* and *in vivo* data, we assume that telethonin binds to p53 as well as to the E3 ubiquitin ligase MDM2, essentially supporting p53 degradation. Loss of or mutations in telethonin together with an increase in biomechanical stress causes maladaptation, apoptosis and global heart failure.