UNIVERSITY OF CALIFORNIA SAN DIEGO

The Role of NEDD4 in Adult Cardiomyocytes

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in

Biology

by

Tiana Huynh

Committee in Charge:

Professor Ju Chen, Chair
Professor Deborah Yelon, Co-Chair
Professor Emma Farley

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The Thesis of Tiana Huynh is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California San Diego

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DEDICATION

I dedicate this thesis to my parents, Chris and Nanci Huynh, and my brother, Tyler Huynh. I would also like to dedicate this thesis to Dante Noffal, Erica Bui, Gabby Guzman, Esther Suh, Lauryn Johnson, and Elyssa Mejia. Without their continuous love and support, I would not be the person I am today. Thank you for helping me achieve my dreams and for always believing in me.

"Together let us seek the heights."

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LIST OF ABBREVIATIONS

NEDD4: Neuronal precursor cell-expressed developmentally downregulated 4

***Nedd4 icKO*:** *Nedd4* cardiomyocyte-specific knockout

E1: Ubiquitin-activating enzyme

E2: Ubiquitin-conjugating enzyme

E3: Ubiquitin-protein ligase

RING: Really Interesting New Gene

HECT: Homologous to the E6-AP Carboxyl Terminus

C2 domain: Protein structural domain that binds to phospholipids and mediates targeting

WW domain: Trp-Trp domain

PY motif: Proline-rich sequence

PTEN: Phosphatase and tensin homolog

HDM2/MDM2: Human homolog of murine double minute 2

N-Myc: Basic helix-loop-helix protein 37

HER3: Receptor tyrosine-protein kinase erbB-3

SAG: Sensitive to apoptosis gene

Ras: Proto-oncogene; Protein belonging to the class of protein called small GTPase

CNrasGEF: Guanine-nucleotide exchange factor

AMOT: Angiomotin

YAP: Yes-associated protein

TAZ: Transcriptional co-activator with PDZ-binding motif

Sav1/WW45: Salvador homolog 1

LATS2: Large tumor suppressor kinase 2

CD40: Cluster of differentiation 40

TNFR: Tumor necrosis factor receptor

TRAF3: TNFR-associated factor 3

IGF-1/IGF-1R: Insulin-like growth factor 1/Insulin-like growth factor receptor 1

Grb10: Growth factor receptor-bound protein 10

Akt: Protein kinase B

FGFR1: Fibroblast growth factor receptor 1

hNEDD4: Human NEDD4

MTMR4: Myotubularin-related protein 4

PDLIM7: PDZ and LIM domain protein 7

ENaC: Epithelial Na+ channel

ABCB1: ATP Binding Cassette Transporter

MVB: Endosomal/multivesicular body

RNAPII: RNA polymerase II

Rsp5: Version of NEDD4 found in yeast

LC3: Microtubule associated protein 1 light chain 3

SQSTM1/p62: Sequestosome-1

Tsp-1: Thrombospondin-1

I/R: Ischemia/Reperfusion

aMHC: Mouse cardiac-specific alpha-myosin heavy chain

MerCreMer: Mutated estrogen receptor flanking Cre recombinase

qRT-PCR: Quantitative real-time polymerase chain reaction

ANF: Atrial natriuretic factor; Cardiac stress marker

BNP: Brain natriuretic peptide; Cardiac stress marker

Coll1a1: Collagen 1a1; Profibriotic marker

Coll3a1: Collagen 3a1; Profibrotic marker

LV: Left ventricle

RV: Right ventricle

BW: Body weight

TL: Tibia length

H&E: Hematoxylin and eosin

LVIDd: Left ventricle internal dimension in diastolic

LVIDs: Left ventricle internal dimension in systolic

LVPWd: Left ventricle posterior wall thickness

FS: Fractional shortening

HSPs: Heat shock proteins

sHSPs: Small heat shock proteins

Hsp90: Heat shock protein 90

Hsp60: Heat shock protein 60

Hsp22/HspB8: Small heat shock protein family B member 8

Hsp20/HspB6: Small heat shock protein family B member 6

BAG3: Chaperon protein; Bcl-associated athanogene 3

TAM: Tamoxifen

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ABSTRACT OF THE THESIS

The Role of NEDD4 in Adult Cardiomyocytes

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Tiana Huynh

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Professor Ju Chen, Chair
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Neuronal precursor cell-expressed developmentally downregulated 4 (NEDD4) is a member of the NEDD4 family of HECT E3 ubiquitin ligases. NEDD4 is highly expressed in cardiomyocytes, but its specific function in adult cardiomyocytes is unknown. To study the role of NEDD4 in adult cardiomyocytes, we have generated a *Nedd4* floxed mouse model and crossed it with tamoxifen-inducible aMHC-MerCreMer mice to generate an inducible *Nedd4* cardiomyocyte-specific knockout mouse model (*Nedd4* icKO). In this study, we performed comprehensive molecular and cardiac physiological studies on *Nedd4* icKO mice. Our results show that after performing age-dependent studies, cardiac function and structure were not

dramatically altered in *Nedd4* icKO mice, suggesting that the loss of NEDD4 did not cause cardiomyopathy. Further experiments revealed that the levels of chaperone proteins and the levels of global ubiquitination were not changed within the cardiac tissue of *Nedd4* icKO mice. Together, these results suggest that deletion of NEDD4 in adult cardiomyocytes does not cause overt cardiomyopathy.

INTRODUCTION

This project examines the role of neuronal precursor cell-expressed developmentally downregulated 4 (NEDD4) in cardiac disease and function as it pertains to adult cardiomyocytes.

Ubiquitin proteasome pathway and protein quality control:

The ubiquitin proteasome pathway is responsible for protein quality control in eukaryotic cells. More specifically, ubiquitylation is a type of protein modification that leads to protein degradation, endocytosis, and the sorting and trafficking of transmembrane proteins.[1] It involves the sequential transfer of activated ubiquitin between a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin-protein ligase (E3).[1] E3 ubiquitin ligases play a role in this process by transferring ubiquitin from E2 to Lys residues on the enzyme's substrate, which can become monoubiquitinated or polyubiquitinated.[1] Ubiquitin contains seven Lys residues itself, with Lys48 and Lys63 being the most commonly used.[1] The type of ubiquitin chains formed between ubiquitin's Lys residues and its substrate's residues determines the substrate's destination or fate within the cell.[1] For example, Lys48-linked ubiquitin chains are typically degraded by the 26S proteasome, while Lys63-linked ubiquitin chains are involved in various cellular processes.[1] Because E3 is largely responsible for the specificity of ubiquitylation, organisms typically contain a large number of E3s and very little E1s and E2s.[1] There are two main classes of E3 ligases: the RING (Really Interesting New Gene) E3 and the HECT (Homologous to the E6-AP Carboxyl Terminus) E3.[2] NEDD4 is a member of the NEDD4 family of HECT E3 ubiquitin ligases.

NEDD4's structure as a HECT E3 ubiquitin ligase:

The HECT family of ubiquitin ligases is comprised of 28 proteins and contains approximately 350 amino acids within its domain.[2] HECT E3 ubiquitin ligases are responsible for dictating the specificity of ubiquitylation and regulating the trafficking of receptors, channels, transporters, and viral proteins.[1] They do this by accepting ubiquitin from an E2 and then transferring it to its substrate.[2] The NEDD4 family of HECT E3 ubiquitin ligases has nine members: NEDD4, NEDD4-2 (NEDD4L), ITCH, SMURF1, SMURF2, WWP1, WWP2, HECW1, and HECW2. NEDD4 is comprised of an N-terminal C2 domain that binds to phospholipids and mediates targeting; 2-4 WW (Trp-Trp) domains that bind to proline-rich, or PY, motifs on its substrate; and a C-terminal HECT domain that contains its catalytic, or ubiquitin transfer, activity (Figure 1A).

The substrates of NEDD4:

NEDD4 has been found to be correlated with substrates that are related to cancers. One of these substrates is the phosphatase and tensin homolog (PTEN), a tumor suppressor that is often mutated or deleted in various human cancers.[3] It has previously been found that NEDD4 negatively regulates PTEN by poly-ubiquitylating and degrading it within the cell.[4] NEDD4 is capable of interacting with PTEN by binding to PTEN through its C2, or HECT domain.[4] Another one of these substrates is the human homolog of murine double minute 2 (HDM2 or MDM2). MDM2 is capable of negatively regulating the tumor suppressor gene p53 and the overexpression of MDM2 can inactivate p53.[4] It has been found that NEDD4 promotes Lys63-type poly-ubiquitylation of MDM2 via its RING domain.[4] Basic helix-loop-helix protein 37 (N-

Myc) is another proto-oncogene associated with NEDD4. N-Myc is a member of the Myc transcription factor family and its dysregulation leads to the development of several tumors.[4] Previously, it had been discovered that NEDD4 directly binds to N-Myc in the nucleus. This binding allows NEDD4 to be able to negatively regulate N-Myc's protein stability by increasing its poly-ubiquitylation.[4] Additionally, receptor tyrosine-protein kinase erbB-3 (HER3), which is a member of the epidermal growth factor family and plays a role in increasing cell migration and proliferation with regards to cancer, was found to be another substrate of NEDD4.[4,5] It was found that the C-terminal tail of HER3 interacted with the WW domains of NEDD4 and that the knocking down of NEDD4 led to the increase of HER3 signaling and cancer proliferation both *in vivo* and *in vitro*.[5] It has also been found that the sensitive to apoptosis gene (SAG), which protects cells from apoptosis caused by various stimuli and is required for lung tumorigenesis, is another substrate of NEDD4.[6] In the study, it was discovered that NEDD4 and SAG interact with each other through the HECT domain and RING domain, respectively, and that both of these domains are required for the ligase activity of either protein.[6] Another proto-oncogene found to interact with NEDD4 is Ras, whose mutations are found in approximately 30% of human cancers.[4] Ras signaling leads to the transcription of NEDD4, which then mediates Ras levels. When Ras proteins are overactivated, it prevents NEDD4 from ubiquitinating and degrading Ras, allowing Ras to avoid regulation by NEDD4 and may be the cause behind the formation of tumors.[4] Another member of the Ras family, guanine-nucleotide exchange factor, CNrasGEF, was found to be ubiquitinated by NEDD4 through its PY motif binding to the WW domains of NEDD4.[4] Although the role of CNrasGEF in cancer biology is complicated, it has been found to play roles in skin and brain cancers.[4]

Additionally, NEDD4 has also been seen to interact with proteins involved in the Hippo pathway, which plays a role in controlling organ size by regulating cell proliferation, apoptosis, and stem cell self-renewal.[7] Dysregulation of this pathway can also ultimately lead to cancer.[7] One of these proteins is angiomotin (AMOT), which is a membrane-associated protein responsible for migration, tight junction formation, cell polarity, and angiogenesis and plays a significant role in the Hippo pathway by regulating the subcellular localization of Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ), two co-activators in the pathway.[8] Previously, it has been found that AMOT undergoes proteasomal degradation with NEDD4 being the E3 ligase for AMOT/p130, the long isoform of AMOT.[8] It was found that the L/P-PXY motifs of AMOT/p130 and the WW domains of NEDD4 interact with each other to mediate this interaction.[8] It was also found that the proteins salvador homolog 1 (SAV1 or WW45) and large tumor suppressor kinase 2 (LATS2), which are needed to activate the Hippo pathway, are ubiquitinated by NEDD4.[9] WW45 was seen to bind to NEDD4 through its N-terminal domain, which then leads to WW45's degradation.[9] With regards to LATS2, it was found that NEDD4 binds to LATS2 mostly through amino acids 281-660 of its WW domain, which then also leads to LATS2 degradation.[9]

NEDD4 has also been found to be involved in the CD40 signaling pathway. Cluster of differentiation 40 (CD40) is a member of the tumor necrosis factor receptor (TNFR) superfamily and plays an important role in B-cell mediated immunity.[10] CD40 engagement induces the assembly of the CD40 signaling pathway, which then leads to multiple downstream events required for CD40 function.[10] One of the known components of this pathway is TNFR-associated factor 3 (TRAF3), which binds to CD40 and mediates downstream signaling.[10] It was found that

when TRAF3 is recruited to the CD40 complex, NEDD4 ubiquitinates TRAF3 via Lys63-ubiqutination, which is critical for CD40-mediated Akt activation and the regulation of B-cell mediated immunity.[10]

Another pathway that NEDD4 has been found to take part in is the insulin-like growth factor 1 (IGF-1) pathway. IGF-1 is a growth hormone and was determined to be another one of NEDD4's substrates.[11] However, NEDD4 doesn't directly bind to insulin-like growth factor receptor 1 (IGF-1R). They are instead linked together by the adaptor known as growth factor receptor-bound protein 10 (Grb10), which allows IGF-1R to then be ubiquitinated and eventually degraded.[11] It was found that *Nedd4*-null mice not only resulted in decreased levels of IGF-1 but also reduced insulin signaling, delayed embryonic development, reduced growth, reduced body weight, and led to neonatal fatality.[11] Based on this study, it was determined that NEDD4 is capable of controlling IGF-1 and insulin signaling through Grb10. Similarly, NEDD4 also targets protein kinase B (Akt) for phosphorylation and trafficking within the IGF-1 pathway.[4] Akt is an enzyme that plays a key role in regulating metabolism, cell survival, motility, transcription, and cell-cycle progression.[4] It was found that the HECT domain of NEDD4 binds to the PH domain of Akt, which promotes the Lys63-ubiquitination of Akt.[4]

Another growth factor receptor NEDD4 targets is fibroblast growth factor receptor 1 (FGFR1), which plays a role in cell proliferation and differentiation with regards to adult development and homeostasis.[12] Human NEDD4 (hNEDD4) is able to regulate the endocytosis and signaling of FGFR1 by binding to FGFR1's non-canonical motif, FGFR1-Δ6, via its WW3

domain.[12] Deletion of FGFR1-Δ6 ultimately prevents hNEDD4 binding and ubiquitination.[12] However, this relationship only occurs between hNEDD4 and FGFR1 because of hNEDD4's WW3 domain, which is not present in rat NEDD4 or hNEDD4-2.[12]

NEDD4 has also been found to be correlated with substrates with regards to muscle atrophy. One of these proteins is myotubularin-related protein 4 (MTMR4), an inositol phosphatase containing a PY motif within skeletal muscles.[13] MTMR4 plays a role in endosomal trafficking and was found to interact with NEDD4 in a WW domain-PY motif interaction.[13] By using a rat model for muscle denervation atrophy, the study found an increase in the levels of NEDD4 and a decrease in levels of MTMR4 in the atrophying muscle, suggesting NEDD4 ubiquitinates MTMR4 and that MTMR4 may play a role in NEDD4-mediated skeletal muscle atrophy.[13] In addition, PDZ and LIM domain protein 7 (PDLIM7), a protein known to regulate the development and function of muscles, was found to interact with NEDD4.[14] It was found that the PY motif of PDLIM7 binds to the second and third WW domains of NEDD4 exogenously, while endogenously they were found to interact with each other in the cytoplasm of myotubes, ultimately leading to the ubiquitination of PDLIM7.[14]

NEDD4 was also found to antagonize Notch within the Notch signaling pathway using a *Drosophila* model.[15] The Notch gene encodes for a cell surface receptor that allows cell-cell communication and homeostasis and cell development regulation.[4] It was found that NEDD4 binds to Notch's PPSY motif, which results in its ubiquitination and helps control its intracellular

localization.[15] It is thought that NEDD4 binds to Notch in order to inactivate and prevent its sporadic activation at the cell surface.[15]

The epithelial Na+ channel (ENaC) was also found to interact with NEDD4 and mutations in this channel have been found to cause Liddle's syndrome, an inherited form of high blood pressure.[16] These mutations involve the C terminus of the beta or gamma ENaC subunits and lead to the deletion of its PY motif, which interacts with NEDD4.[16] Inability to interact with NEDD4 prevents inhibition of the Na+ channel, allowing Na+ to be more readily absorbed since NEDD4 is no longer able to degrade the channels located on the cell surface.[16] It is thought that this loss of interaction is the cause behind Liddle's syndrome and other forms of hypertension.[16]

Additionally, NEDD4 was found to interact with substrates related to neurodegenerative diseases. One of these substrates is the ATP Binding Cassette Transporter (ABCB1), which exports the peptide B-amyloid from endothelial cells that line the blood-brain barrier.[17] However, in Alzheimer's disease, the amount of ABCB1 at the blood-brain barrier is decreased and it was found that the ubiquitin E3 ligase responsible for the density of ABCB1 at the barrier's surface is NEDD4.[17] Lack of ABCB1 was correlated to an increase of NEDD4 in mice brain capillaries *ex vivo* and mass spectrometry revealed eight lysine residues that were ubiquitinated by NEDD4.[17] More specifically, all of the lysine residues found were located on the surface of the intracellular domains of ABCB1 with three of these residues located inside the intracellular loop of a transmembrane helix close to a putative NEDD4 binding site.[17] Another substrate of NEDD4 is a-synuclein, a neuronal protein found in Lewy bodies associated with Parkinson's disease.[4] a-

synuclein possess a PY motif within its C terminus that interacts with NEDD4.[4] It was found that NEDD4 is highly expressed in neurons with Lewy bodies and a downregulation of NEDD4 reduces the degradation of a-synuclein, indicating that an increase in NEDD4 may be a solution that could protect against the pathogenesis of Parkinson's disease.[4]

Another substrate of NEDD4 is gamma2-Adaptin, a member of the clathrin adaptor protein family, which help mediate the sorting of cargo throughout the cell.[18] gamma2-Adaptin is able to act as a ubiquitin receptor because of its ubiquitin-interacting motif, which binds to the C2 domain of NEDD4.[18] This interaction leads to the mono- and poly-ubiquitination of gamma2-Adaptin. It was later discovered that gamma2-Adaptin functions in the endosomal/multivesicular body (MVB) pathway since depletion of gamma2-Adaptin resulted in deformed MVB vesicles, which could not be rescued by a homolog of gamma2-Adaptin.[18] The inability of the vesicles to be rescued was due to the homolog's lack of a ubiquitin-interacting motif, which suggests NEDD4 and gamma2-Adaptin, whose function remained largely unknown, may operate within the MVB pathway.[18]

NEDD4 was also found to interact with RNA polymerase II (RNAPII). RNAPII is responsible for initiation, elongation, and termination when transcribing the protein-encoding genes of mRNA.[4] NEDD4 ubiquitinates and degrades Rpb1, RNAPII's largest subunit, when DNA becomes damaged by UV-light.[4] This relationship between NEDD4 and RNAPII became elucidated when reduced NEDD4 levels resulted in lower levels of RNAPII ubiquitination. However, this was restored when the activity of NEDD4 was increased.[4] Within yeast, NEDD4

is referred to as Rsp5, and it was discovered that Rsp5 produces a mixture of mono-ubiquitinated and Lys63-linked poly-ubiquitinated RNAPII.[4] However, only the mono-ubiquitinated RNAPII is capable of being degraded.[4]

Lastly, NEDD4 has been seen to be associated with autophagy, a lysosomal-mediated cellular degradation process that relieves cells under stress.[19] One of the proteins associated with autophagy is Beclin 1, with increased levels of Beclin 1 being correlated with the prolonged survival of tumor cells by most likely enhancing autophagy while preventing apoptosis.[4] It has been found that Beclin 1 contains a PY-like motif, which interacts with the WW domains of NEDD4.[4] Once bound to Beclin 1, NEDD4 controls its poly-ubiquitination and degradation, allowing for the inhibition of autophagy.[4] Another protein associated with autophagy is microtubule associated protein 1 light chain 3 (LC3), which recruits autophagic proteins to autophagosomes, and has been found to interact with NEDD4 and sequestosome-1 (SQSTM1/p62), an autophagic cargo receptor.[19] Previously, it had been discovered that NEDD4 interacts with LC3 through its LC3-interacting region domain and that downregulation of NEDD4 decreased LC3 levels but increased SQSTM1 levels.[4] However, upon further inspection, it was discovered that LC3 is an activator, not a substrate of NEDD4, while SQSTM1 is a Lys63-linked poly-ubiquitinated substrate of NEDD4.[4,19] NEDD4 is thought to interact with the PB1 domain of SQSTM1 via its HECT domain.[4]

The cardiac role of NEDD4:

NEDD4 is highly expressed in adult cardiomyocytes, but its function remains largely unknown. Previously, it had been determined that the global knockout of *Nedd4* in mice led to embryonic lethality at mid-gestation.[20]The embryos expressed severe heart defects and vascular abnormalities, specifically double-outlet right ventricle and atrioventricular cushion defects.[20] It was also discovered that upon the knocking out of *Nedd4,* Thrombospondin-1 (Tsp-1) was increased.[20] Tsp-1 is an inhibitor of angiogenesis, which is the development of new blood vessels. This suggests that NEDD4 is a suppressor of Tsp-1 and that the increased levels of Tsp-1 may be the cause of the embryonic heart defects that were found.[20] As a result, this study suggests NEDD4 may play a significant role in embryonic development and cardiac development. It has also been determined that NEDD4 regulates the nuclear trafficking of the active form of Akt.[4] Activation of the Akt pathway has shown to protect the heart against ischemia/reperfusion (I/R) injury, which occurs when the blood supply to the heart has been cut off.[21] It was found that after I/R, NEDD4 levels were decreased in both rat heart tissue as well as H9C2 cardiomyocytes.[21] However, overexpression of NEDD4 *in vitro* and *in vivo* activated the Akt pathway and reduced myocardial apoptosis after I/R.[21] These results suggest NEDD4 is able to protect the myocardium from I/R induced apoptosis by activating the Akt pathway. However, the role of NEDD4 in adult cardiomyocytes as it pertains to cardiac disease and function still remains unknown. Thus, this study aims to investigate whether or not the loss of NEDD4 results in cardiomyopathy in adult mice by generating an inducible *Nedd4* cardiomyocyte-specific knockout mouse model (*Nedd4* icKO).

MATERIALS AND METHODS

Animal Protocol and Consent

All mice used in this study were maintained by the UCSD Animal Care Program within the vivarium of Biomedical Research Facility II. Housing of the rodents followed the University of California, San Diego's Institutional Animal Care and Use Committee (IACUC) guidelines with enrichment being provided in standard light/dark cycle conditions. All experimental procedures and protocols were approved by IACUC.

Mouse Models

Using a tamoxifen-inducible Cre-Lox recombination method, a *Nedd4* floxed mouse model was crossed with tamoxifen-inducible aMHC-MerCreMer mice to generate an inducible *Nedd4* cardiomyocyte-specific knockout mouse model (*Nedd4* icKO) (Figure 1, B and C). aMHC, or alpha-myosin heavy chain is a cardiac-specific promoter that allows for the targeted deletion of *Nedd4* in only cardiomyocytes, and Mer, or mutated estrogen receptor, binds to tamoxifen and allows for the temporal activation of Cre recombinase within the Cre-Lox recombination method. Both the *Nedd4* floxed mouse model and the tamoxifen-inducible aMHC-MerCreMer mice were generated within the Dr. Ju Chen Laboratory and available to be crossed to produce the desired mouse model. Genotyping was performed as detailed in previous publication with primers indicated in Table 1 (Figure 1C).[22] From there, 8-week old NEDD4 flox/flox; αMHC-MerCreMer+ (icKO) and NEDD4 Flox/Flox; Cre-control (Ctrl) mice were injected peritoneally with tamoxifen at a dosage of 40ug/kg/day for three consecutive days (Figure 1B). To account

for variation in different backgrounds, the control and knockout mice were generated and maintained in a pure C57/B6 background and biological variables, such as age, sex, and weight were accounted for. The *Nedd4* control and icKO mice were observed at various different time points after tamoxifen injection.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from mouse left ventricles using TRIzol reagent (Life Technologies, Thermo Fisher Scientific) per manufacturer's recommendations.[22] cDNA was synthesized using MMLV Reverse Transcription (Promega).[22] Primer sequences for qRT-PCR can be found in Table 2. (IDT) qRT-PCR reactions were performed using iTaq Universal SYBR Green Supermix in 96-well, low profile PCR plates in a Bio-Rad CFX96 Thermocycler.

Protein Isolation and Western Blot Analysis

Total protein extracts were prepared by suspending ground heart tissue in urea lysis buffer (8M urea, 2M thiourea, 3% SDS, 75mM DTT, 0.03% bromophenol blue, 0.05M Tris-HCl, pH 6.8).[22] Protein lysates were then separated using Bolt 4-12% Bis-Tris Plus gels (Life Technologies, Thermo Fisher Scientific) and transferred overnight onto PVDF membranes (Bio-Rad) at 4°C. The membranes were then blocked in TBS containing 0.1% Tween-20 (TBST) and 5% dry milk for 1 hour and incubated overnight at 4°C with the indicated primary antibodies.[22] The membranes were washed and incubated for 1 hour at room temperature with HRP-conjugated secondary antibodies generated in rabbit (1:3000) or mouse (1:3000) (DAKO). Immunoreactive

protein bands were then visualized using Clarity Western ECL Substrate (Bio-Rad). Antibodies can be found in Table 3.

Histology

Hearts were isolated from age- and sex-matched littermates, washed in PBS, and fixed overnight in 4% paraformaldehyde (PFA).[22] The hearts then underwent a sucrose gradient of 5% sucrose, 10% sucrose, and 15% sucrose for 4 hours or up to overnight at 4℃. The hearts were then placed in 20% sucrose overnight at 4℃. Next, the hearts were embedded in 1:1 OCT:20% sucrose for 4 hours or up to overnight at 4℃ and coronally sectioned (10-um thickness). Sections were then stained with Hematoxylin and eosin (H&E), mounted, and then imaged using a Hamamatsu NanoZoomer 2.0HT Slide Scanning System.

Echocardiography

Mice were briefly anesthetized with 1% isoflurane and underwent echocardiography using FUJIFILM VisualSonics SonoSite Vevo 2100 ultrasound system with a 32- to 55-MHz linear transducer.[22] The percentage of fractional shortening (FS) was used as an indicator of systolic cardiac funciton.[22] Measurements of heart rate, left ventricular internal dimension systolic (LVIDs), left ventricular internal dimension diastolic (LVIDd), and left ventricular posterior wall thickness (LVPWd) were determined from the LV M-mode tracing.[22]

RESULTS

NEDD4 mRNA levels and protein levels are decreased in the cardiac tissue of *Nedd4* icKO mice.

To examine the efficiency of our generated *Nedd4* icKO mouse model, NEDD4 mRNA levels of both *Nedd4* control and icKO mice 2 weeks post-tamoxifen injection were measured through quantitative real-time polymerase chain reaction (qRT-PCR). *Nedd4* icKO mice showed a decrease in NEDD4 mRNA levels when compared to that of *Nedd4* control mice 2 weeks post-tamoxifen injection (Figure 2A). To further confirm this deficiency, Western blot was also performed on protein lysates from *Nedd4* control and icKO mice from 2 and 5 weeks post-tamoxifen injection. As expected, the Western blots and their respective quantifications also revealed a decrease in protein levels in both groups of *Nedd4* icKO mice when compared to the levels of *Nedd4* control mice (Figure 2, B-E). Additionally, the loss of NEDD4 did not lead to any statistically significant changes in gene expression levels for all other NEDD4 family members at 2 or 5 weeks post-tamoxifen injection (Figure 3). This indicates that the other NEDD4 family members did not compensate for the loss of NEDD4 within adult cardiomyocytes.

From here, a group of *Nedd4* control and icKO mice were observed immediately following tamoxifen injection and a survival curve was generated to ensure the mice model could be aged and studied up to our desired time points. Approximately 10% of *Nedd4* icKO mice were found dead 50 days post-tamoxifen injection, with a total of 40% being found dead 350 days post-tamoxifen injection at the end of the study. With regards to *Nedd4* control mice, 0%

were found dead at 50 days post-tamoxifen injection, with a total of 30% being found dead at the end of the study. Based on the survival data, the loss of NEDD4 in adult cardiomyocytes is not considered to be lethal (Figure 4).

NEDD4 deficiency in adult cardiomyocytes leads to elevated cardiac stress markers.

To examine any cardiac stress that may be present in *Nedd4* icKO mice, atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP) levels were measured through qRT-PCR. ANF and BNP are members of the natriuretic peptide family that are produced and secreted from the atria.[23] They play an important role in maintaining cardiac homeostasis and display increased levels when the heart is under stress from external pressures and serve as strong indicators of cardiac stress.[23] As expected, *Nedd4* icKO mice 2 weeks and 5 weeks post-tamoxifen injection show an increase in both ANF and BNP levels when compared to the levels of *Nedd4* control mice, with there being a significant increase at 5 weeks post-tamoxifen injection (Figure 4, A-B and E-F). This indicates the homeostasis of the heart has been disrupted. In addition, cardiac fibrosis was also measured through the profibrotic genes collagen type 1 alpha 1 (Collagen 1a1 or Col1a1) and collagen type 3 alpha 1 (Collagen 3a1 or Col3a1) via qRT-PCR. Fibrosis is the development of fibrous connective tissue in response to some form of injury in the heart.[24] Neither Col1a1 nor Col3a1 indicated a significant change in gene expression levels between *Nedd4* control and icKO mice 2 weeks and 5 weeks post-tamoxifen injection (Figure 5, C-D and G-H). This suggests fibrosis has not yet been activated at these time points.

NEDD4 deficiency does not result in changes to cardiac structure and function.

To examine the overall cardiac phenotype, heart tissue was collected at 4, 28, and 36 weeks post-tamoxifen injection. The left ventricle (LV) and right ventricle (RV) were separated from whole heart tissue and measured against the body weight (BW) and tibia length (TL) of the mice. *Nedd4* icKO mice displayed RV/BW and LV/BW ratios that were comparable to that of *Nedd4* control mice at all time points (Figure 6, A and B). To further support these findings, the heart weight was then compared to the tibia length of the mice. Tibia length provides a more accurate quantification for cardiac hypertrophy since body weight can fluctuate during the aging process, while tibia length remains constant after maturity.[25] *Nedd4* icKO mice again displayed RV/TL and LV/TL ratios that were comparable to that of the control mice at all time points (Figure 6, C and D). These results indicate that *Nedd4* icKO mice do not statistically differ in cardiac size when compared to the control group.

To further examine any phenotypic changes caused by the loss of NEDD4, the morphology of the heart was examined through histological studies at 4, 12, 24, and 52 weeks post-tamoxifen injection. For all the time points, the studies revealed no dramatic change in whole heart size between the *Nedd4* icKO mice and control mice when examined using a microscope (Figure 7, A-D (top)). Hematoxylin and eosin (H&E) staining was also performed on these samples as well. Hematoxylin is a bluish dye that stains for basophilic cellular organisms, while eosin is a pinkish dye that stains for eosinophilic cellular organisms.[26] This difference in staining allows for the identification and observation of organelles in great detail.[26] The staining for each time point further supported the images taken via microscope. *Nedd4* icKO mice did not

display an enlarged heart size when compared to that of the control mice (Figure 7, A-C (bottom)). Together with the heart weight to body weight and heart weight to tibia length ratios, *Nedd4* icKO mice do not display cardiac structure that is significantly different to that of *Nedd4* control mice.

To study any changes to cardiac function caused by the loss of NEDD4, age-dependent echocardiographic studies, which utilize an ultrasound that uses sound waves to produce images of the heart, were carried out at 4, 22, and 50 weeks post-tamoxifen injection. After performing these physiological studies, it was revealed the left ventricular internal dimension in diastolic (LVIDd), which measures the left ventricle chamber size when cardiac muscle is relaxed, did not statistically differ between *Nedd4* control and icKO mice at any of the time points (Figure 8A). Additionally, the left ventricular internal dimension in systolic (LVIDs), which measures the left ventricle chamber size when it contracts, did not statistically differ between *Nedd4* control mice and *Nedd4* icKO mice by 50 weeks post-tamoxifen injection (Figure 8B). These results indicate the cardiac chamber size has not changed due to the loss of NEDD4. However, it was discovered that the left ventricular posterior wall thickness (LVPWd) was decreased in *Nedd4* icKO mice at all time points with there being significant differences at 4 and 50 weeks post-tamoxifen injection when compared to that of *Nedd4* control mice, indicating some dilation at the posterior wall (Figure 8C). Additionally, fractional shortening (FS), which measures left ventricle systolic function, was found to be decreased in *Nedd4* icKO mice at all time points, with there being a significant decrease in fractional shortening 4 and 50 weeks post-tamoxifen injection (Figure 8D). This indicates the heart's ability to contract has been compromised. The results of these age-dependent echocardiographic studies further reveal that the loss of NEDD4 in adult

cardiomyocytes does not affect cardiac structure. On the other hand, even though it appears *Nedd4* icKO mice experience some dilation at the posterior wall and contraction has been altered by 50 weeks post-tamoxifen injection, the overall effects caused by the loss of NEDD4 in adult cardiomyocytes have not dramatically affected the cardiac function of the *Nedd4* icKO mice.

NEDD4 deficiency does not result in cardiac stress.

In order to observe the effects that the loss of NEDD4 may have had on the chaperone complex, protein lysates were extracted from the right and left ventricle of *Nedd4* icKO and control mice 2 weeks and 5 weeks post-tamoxifen injection. Heat shock proteins (HSPs) and small heat shock proteins (sHSPs) are chaperones that help facilitate the synthesis of proteins and the refolding and degradation of misfolded proteins, respectively, and are produced by cells in response to stress. Western blot analysis revealed that the levels of Hsp90 and Hsp60, which are ATP-dependent and stress-inducible HSPs, were comparable between the *Nedd4* icKO and control mice at 2 and 5 weeks post-tamoxifen injection (Figure 9, A-D). Furthermore, the protein levels for the ATP-independent small heat shock proteins, Hsp22 (also known as HspB8) and Hsp20 (also known as HspB6), also remained unchanged in the *Nedd4* icKO mice when compared to the control mice 2 and 5 weeks post-tamoxifen injection (Figure 9, A-D) There were also no changes in the levels of BAG3, a Hsp70 co-chaperone, when comparing both groups at 2 weeks and 5 weeks post-tamoxifen injection (Figure 9, A-D). Together, these results indicate that the loss of NEDD4 in adult cardiomyocytes did not cause increased protein stress within the cardiac tissue.

NEDD4 deficiency does not result in global changes to ubiquitin levels.

Since NEDD4 is a HECT E3 ubiquitin ligase that plays a role in the ubiquitin proteasome pathway, it was important to observe if the loss of NEDD4 resulted in any changes to the levels of ubiquitinated proteins within cardiac tissue. In order to study this, protein lysates were isolated from the right and left ventricle of *Nedd4* icKO and control mice at 2 weeks post-tamoxifen injection and from the left ventricle of *Nedd4* and icKO mice at 5 weeks post-tamoxifen injection. Western blot analysis revealed there was no change in levels of ubiquitinated proteins between both the groups at 2 weeks and 5 weeks post-tamoxifen injection (Figure 10, A-D). These results indicate there is no global change in the number of ubiquitinated proteins due to the loss of NEDD4.

DISCUSSION

NEDD4 plays a significant role in the development of the heart. As an E3 ligase, it is a key component within the ubiquitin proteasome pathway that aids in protein degradation, endocytosis, and the sorting and trafficking of transmembrane proteins. It has previously been reported that the global knockout of NEDD4 leads to embryonic lethality, severe heart defects, and vascular abnormalities, while the overexpression of NEDD4 has been found to protect cardiac muscle from ischemia/reperfusion injury.[20,21] Although it has been shown that mutations in NEDD4 can lead to heart defects, little is known with regards to the pathogenesis and molecular mechanism by which the loss of NEDD4 leads to cardiomyopathy.

The generation of our knockout mouse model and its efficiency in tamoxifen-induced gene deletion allows us to examine the functional effects and the molecular mechanism the loss of NEDD4 in adult cardiomyocytes has on mice. Comprehensive molecular and cardiac physiological studies were performed over the course of a year in order to evaluate the long-term effects that the loss of NEDD4 had on cardiac function in *Nedd4* icKO and control mice. Form the survival curve, no premature deaths were found amongst the *Nedd4* mice and by the end of the study, there was no statistical difference in terms of survival rate for both groups. Although the mRNA expression levels of the cardiac fetal markers, ANF and BNP, were increased in the *Nedd4* icKO mice, there was no statistical difference between the knockout group and the control group. This was also true for the profibrotic genes, Coll1a1 and Coll3a1. When examining the heart weight to body weight and heart weight to tibia length ratios, there was no significant difference between the *Nedd4* icKO and control group throughout the entirety of our study. Furthermore, histological studies revealed there were no signs of cardiac hypertrophy, dilation, or fibrosis within the cardiac tissue of *Nedd4* icKO mice when compared to the control group. Together, these results suggest that the loss of NEDD4 did not affect the survivability of the mice nor cause measurable changes in cardiac structure.

The age-dependent echocardiographic studies revealed left ventricular internal dimension diastolic (LVIDd) and left ventricular internal dimension systolic (LVIDs) in *Nedd4* icKO mice to be comparable to that of *Nedd4* control mice. These results indicate that the loss of NEDD4 in adult cardiomyocytes did not result in changes to chamber size. However, by the end of the study at 50 weeks post-tamoxifen injection, *Nedd4* icKO mice showed a decrease in left ventricular posterior wall thickness and fractional shortening when compared to control mice. Although

there was a significant decrease in posterior wall thickness at 50 weeks post-tamoxifen injection, H&E staining needs to be performed on these hearts to determine if there were any phenotypic signs of dilation or any other abnormalities. With regards to the significant decrease in fractional shortening, the heart's ability to properly function is not found to be severely compromised within the *Nedd4* icKO mice since they were still viable at this time point. This suggests that the normal lifespan of mice is not adequate enough to study the full effects of the loss of NEDD4 in adult cardiomyocytes and that this mutation alone may not lead to cardiomyopathy in mice.

Chaperone proteins, such as heat shock proteins (HSPs) and small heat shock proteins (sHSPs), are responsible for protecting proteins when the cell is under some form of stress. In order to examine the affects the loss of NEDD4 had on adult cardiomyocytes at the cellular level, western blot analysis was performed to measure the protein levels of Hsp90, Hsp60, Hsp22, Hsp20, and BAG3. There was no change in protein expression levels for any of these chaperone proteins in the *Nedd4* icKO when compared to the control group. These results suggest that the loss of NEDD4 in adult cardiomyocytes does not cause sufficient cellular damage or stress to induce increased levels of HSPs and sHSPs within the heart.

As an E3 ligase, NEDD4 plays an important role within the ubiquitin pathway by catalyzing the transfer of ubiquitin from the E2 ubiquitin-conjugating enzyme to its substrate. To determine any effects the loss of NEDD4 may have on the levels of ubiquitinated proteins, western blots were performed on protein lysates of *Nedd4* icKO and control mice. It was found that there was no change in levels of ubiquitinated proteins in *Nedd4* icKO mice when compared

to that of the control group. This suggests the loss of NEDD4 does not cause any global changes to the levels of ubiquitinated proteins and that NEDD4 may instead only regulate the ubiquitination of specific proteins. Additionally, it has been discovered that NEDD4 plays a role in another cellular protective process known as autophagy.[19] In future studies, it would be beneficial to perform western blots on autophagosome proteins, such as LC3 and SQSTM1, to determine any effects that the loss of NEDD4 may have on autophagy within the ubiquitin proteasome pathway.

Overall, our observation that the loss of NEDD4 in adult cardiomyocytes does not result in an overt cardiac phenotype or cardiomyopathy is quite unexpected since it is highly expressed in cardiomyocytes. One interpretation behind these results could be due to the lifespan of a mouse not being adequate enough to fully measure the affects that the loss of NEDD4 has on cardiac function and structure. The mice in this study were studied up to 50 weeks post-tamoxifen injection. Future experiments would need to be performed in order to determine whether or not the *Nedd4* icKO mice eventually develop cardiomyopathy with greater age. As of now, however, it is apparent that the loss of NEDD4 in adult cardiomyocytes is not lethal with respect to the mouse's lifespan. However, our present study still provides insight into the function of NEDD4 and can contribute to other studies regarding its role in different developmental stages within cardiomyocytes.

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FIGURES

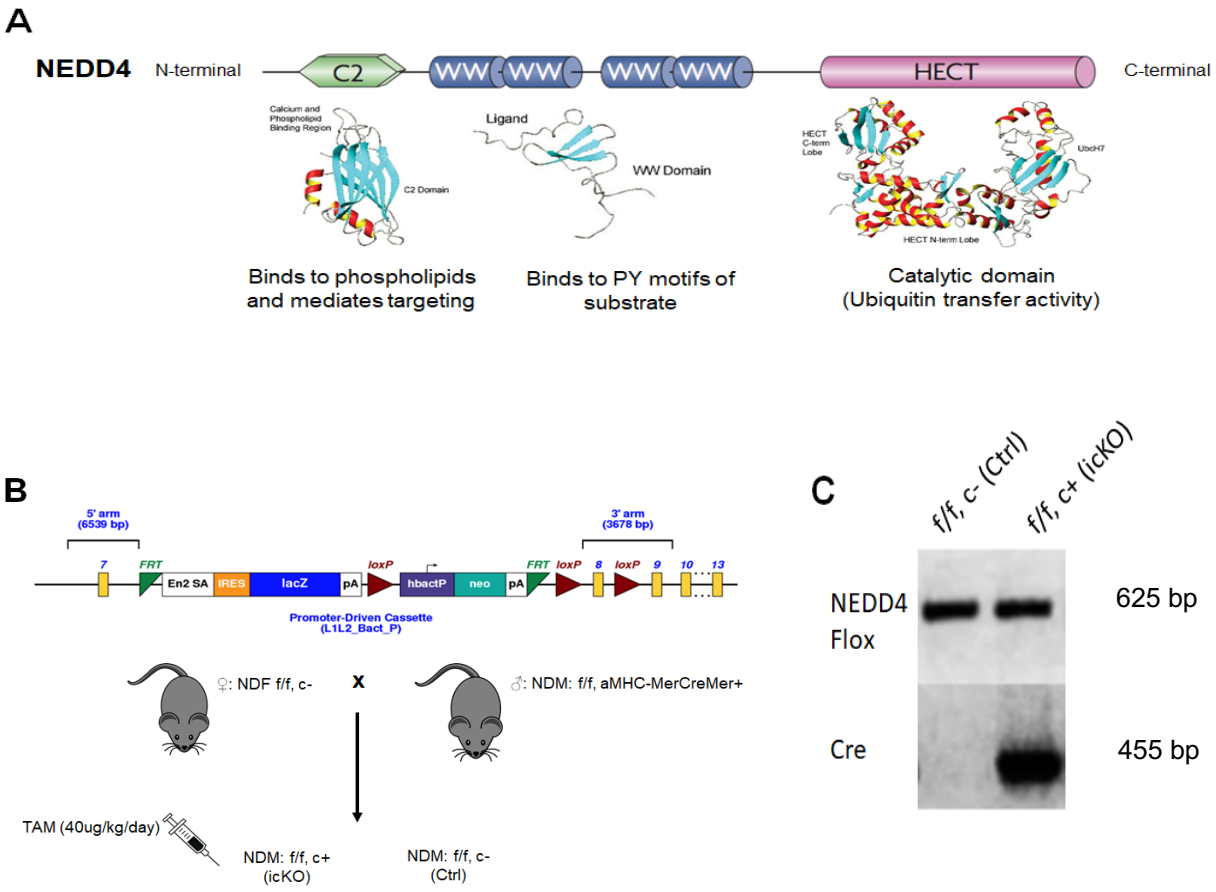

Figure 1. Generation of *Nedd4* icKO and control mice. (A) NEDD4 structure and associated domain structures.[1] (B) Generation of inducible *Nedd4* cardiomyocyte-specific knockout mice using a tamoxifen-induced Cre-Lox recombination method.[27,28,29] (C) Genotyping results of *Nedd4* control and icKO mice.

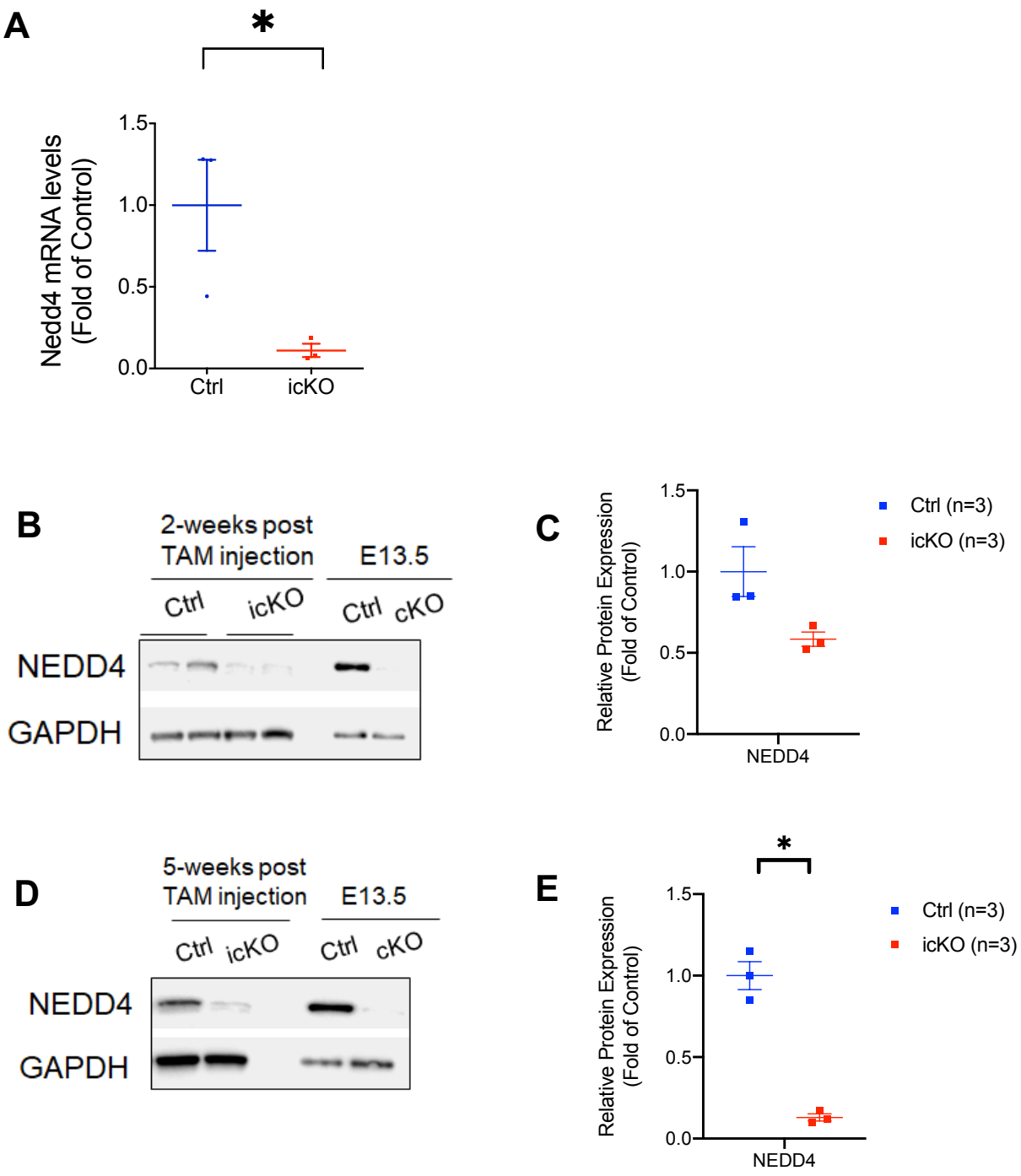

Figure 2. qRT-PCR and Western blot analysis showed decreased levels of *Nedd4* mRNA and protein post-tamoxifen injection. (A) qRT-PCR analysis of *NEDD4* mRNA levels 2 weeks post-tamoxifen injection using primer set 1 (Table 2). (B) Western blot analysis of NEDD4 protein levels 2 weeks post-tamoxifen injection. (C) Quantification analysis for B. (D) Western blot analysis of NEDD4 protein levels 5 weeks post-tamoxifen injection. (E)

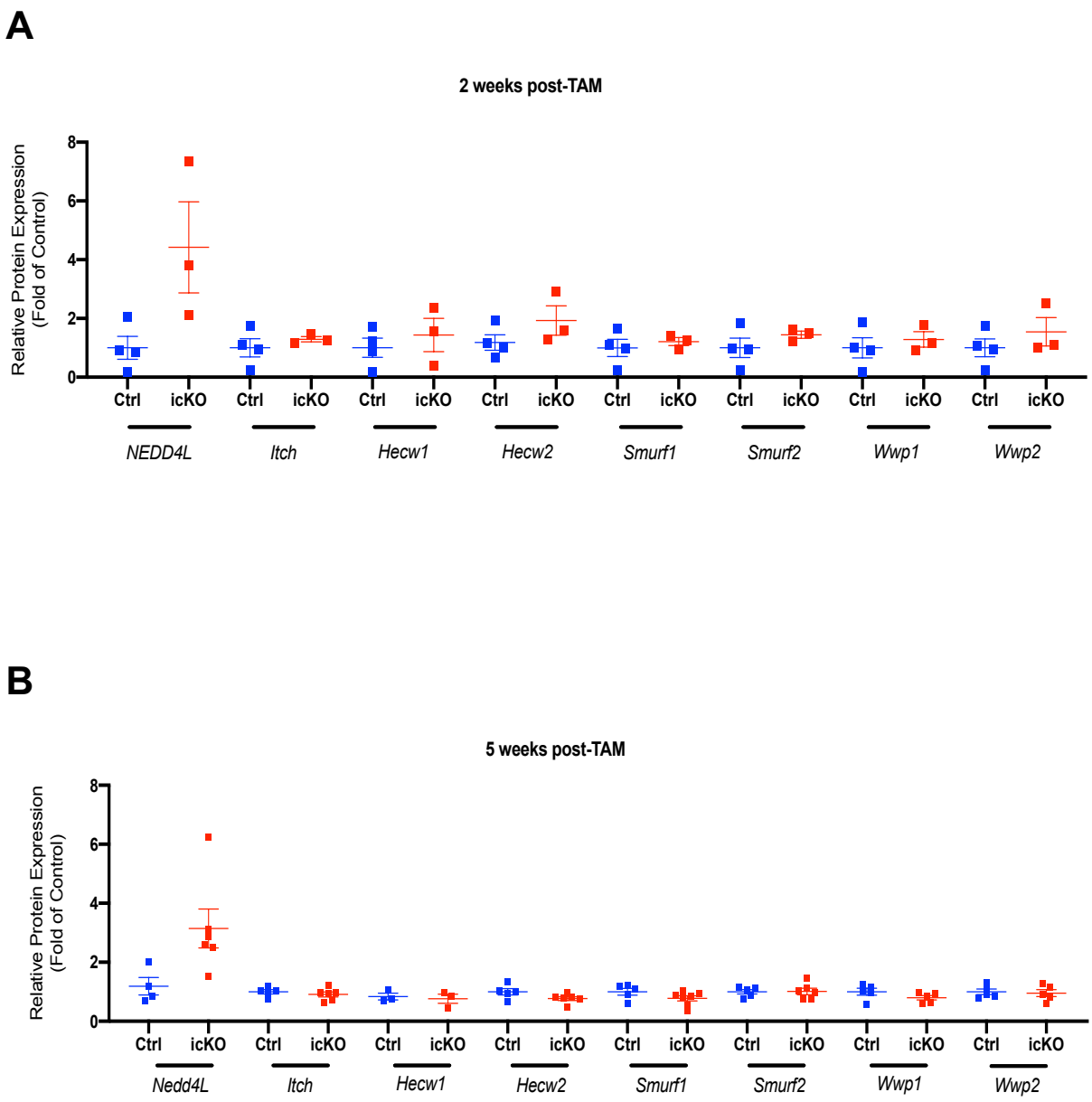

Figure 3. qRT-PCR analysis revealed no significant change in expression levels for other NEDD4 family members in *Nedd4* icKO mice. (A) qRT-PCR analysis of other NEDD4 family members 2 weeks post-tamoxifen injection (Ctrl n=4, icKO n=3). (B) qRT-PCR analysis of other NEDD4 family members 5 weeks post-tamoxifen injection (Ctrl n=4, icKO n=3).

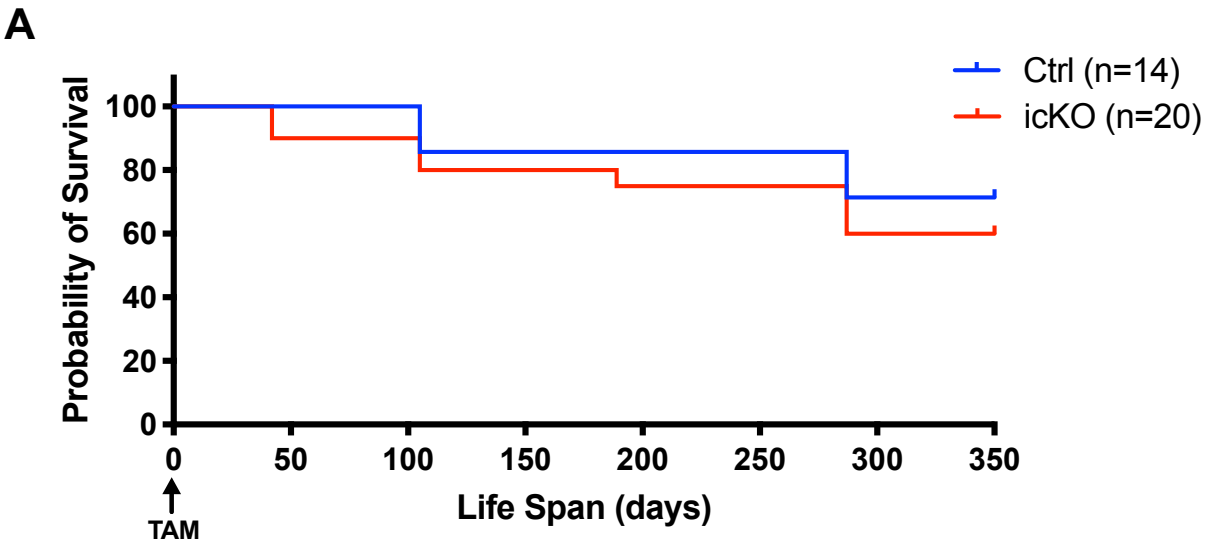

Figure 4. Survival curve of *Nedd4* control and icKO mice post-tamoxifen injection. (A) Survival curve of *Nedd4* control mice (n=14) and *Nedd4* icKO mice (n=20) measured up to 350 days post-tamoxifen injection.

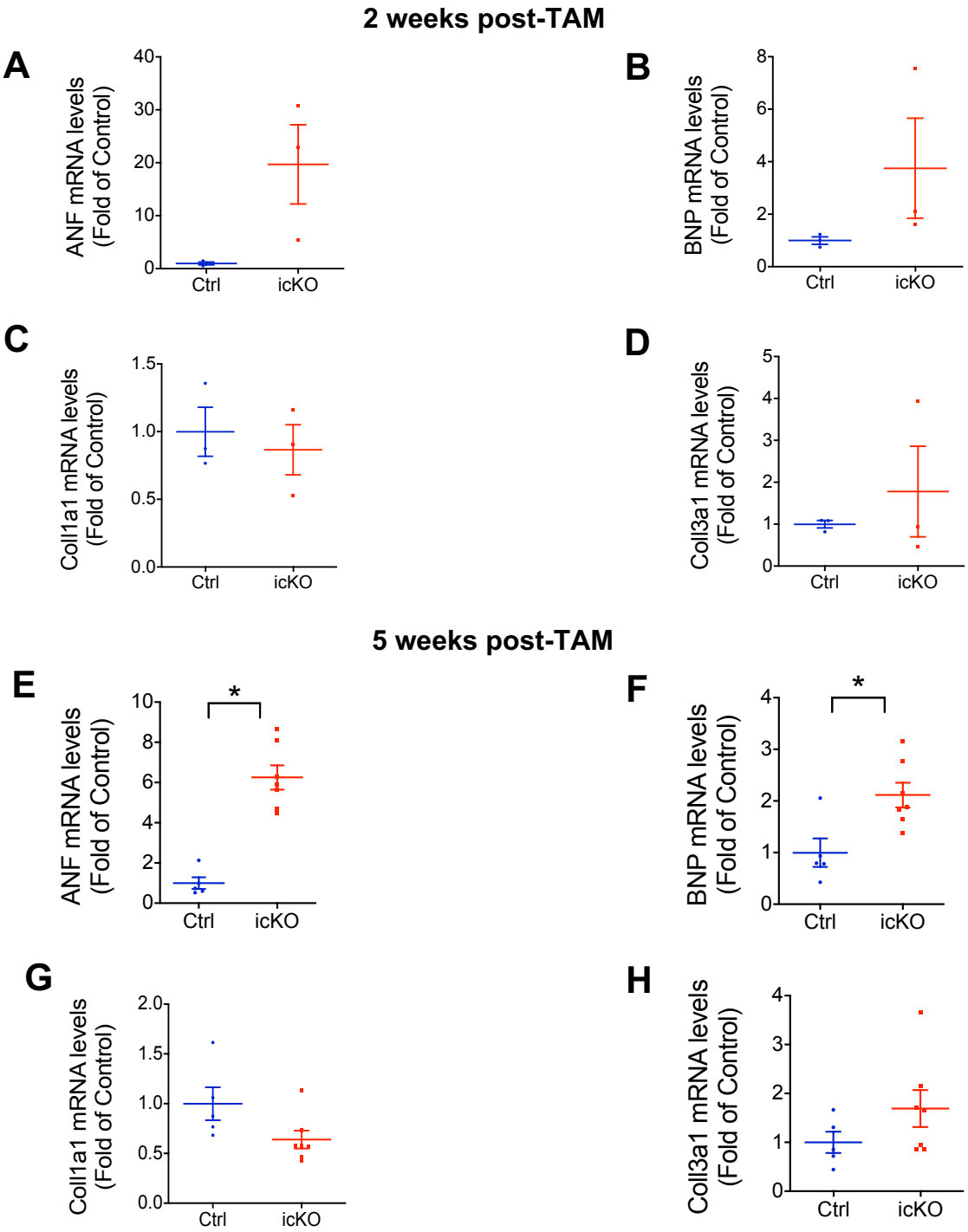

Figure 5. qRT-PCR analysis revealed an increase in cardiac stress markers and no change in cardiac fibrosis markers in *Nedd4* icKO mice. (A-B) qRT-PCR analysis of cardiac stress markers 2 weeks post-tamoxifen injection. (C-D) qRT-PCR analysis of cardiac fibrosis markers 2 weeks post-tamoxifen injection. (E-F) qRT-PCR analysis of cardiac stress markers 5 weeks post-tamoxifen injection. (G-H) qRT-PCR analysis of cardiac fibrosis markers 5 weeks post-tamoxifen injection. (n=3-7 for all groups)

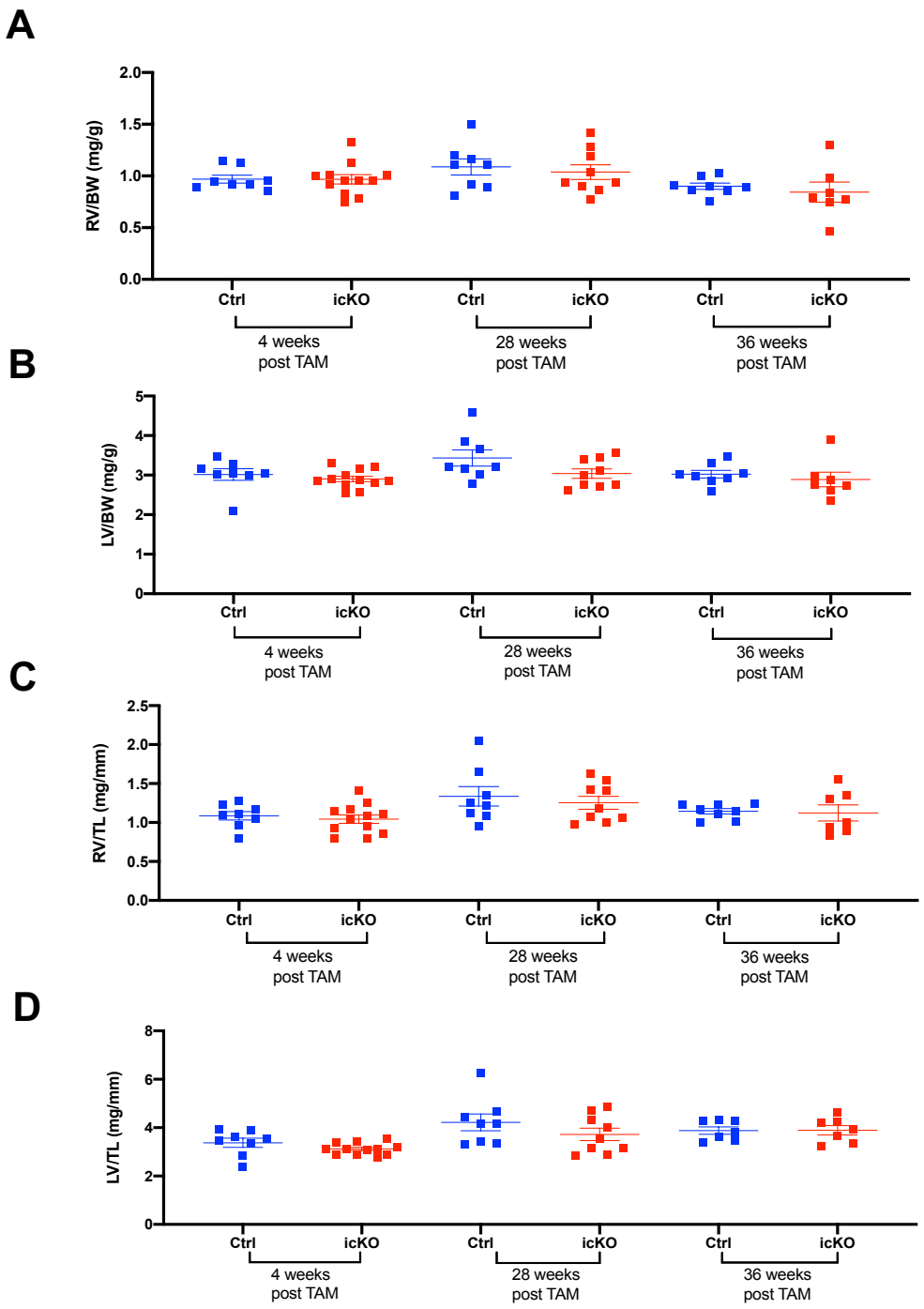

Figure 6. The loss of NEDD4 in adult cardiomyocytes did not result in changes to heart weight in *Nedd4* icKO mice. (A) RV/BW ratios at 4, 28, and 36 weeks post-tamoxifen injection. (B) LV/BW ratios at 4, 28, and 36 weeks post-tamoxifen injection. (C) RV/TL ratios at 4, 28, and 36 weeks post-tamoxifen injection. (D) LV/TL ratios at 4, 28, and 36 weeks post-tamoxifen injection. (n=7-12 for all groups)

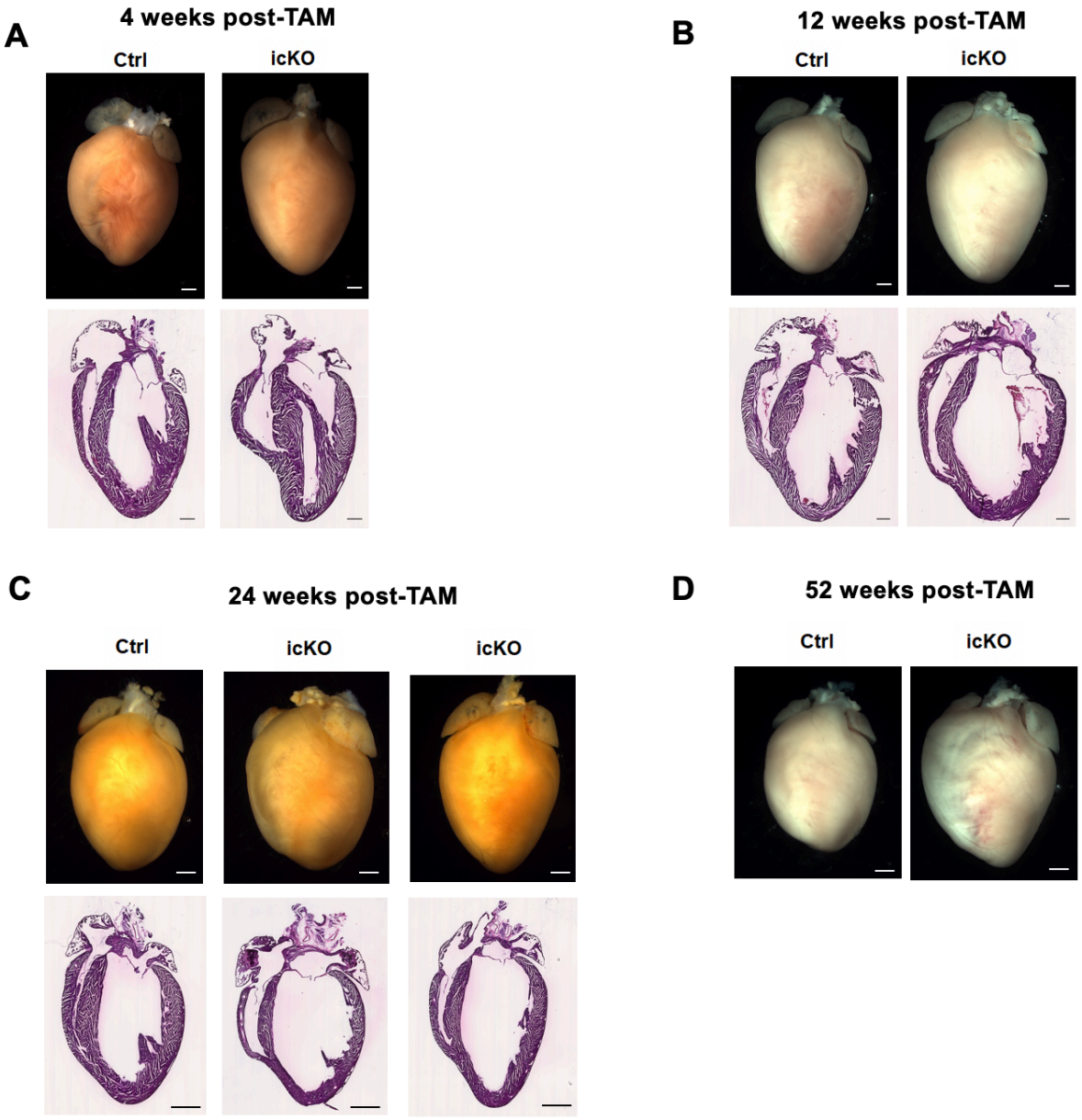

Figure 7. Histological studies revealed no changes to cardiac structure in *Nedd4* icKO mice. (A) Microscopic images (top) and H&E staining (bottom) of *Nedd4* control and icKO mice 4 weeks post-tamoxifen injection. (Scale bar = 1mm) (B) Microscopic images (top) and H&E staining (bottom) of *Nedd4* control and icKO mice 12 weeks post-tamoxifen injection. (Scale bar = 1mm) (C) Microscopic images (top) and H&E staining (bottom) of *Nedd4* control and icKO mice 24 weeks post-tamoxifen injection. (Scale bar = 1mm) (D) Microscopic images of *Nedd4* control and icKO mice 52 weeks post-TAM injection. (Scale bar = 1mm)

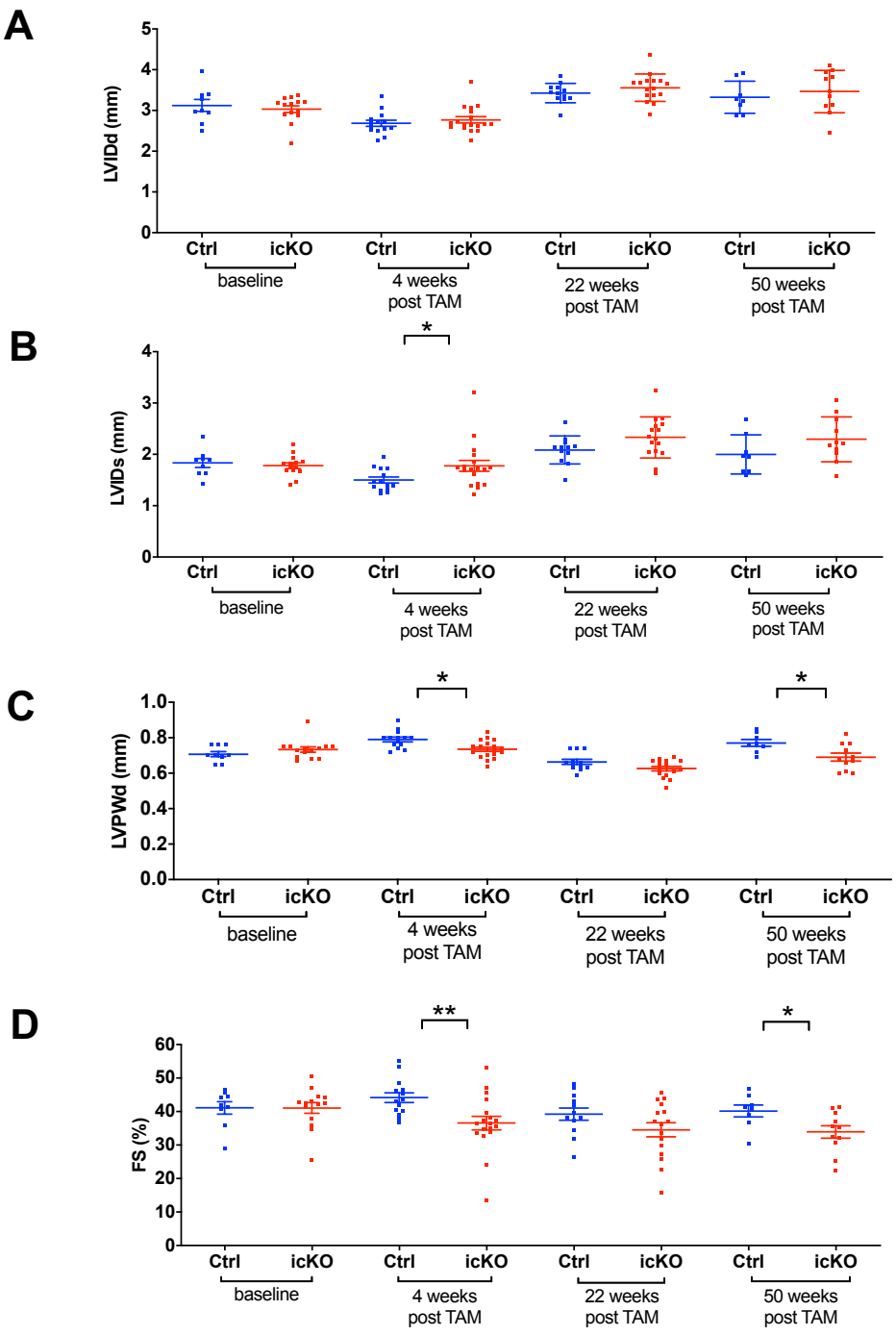

Figure 8. Echocardiographic studies reveal a slight decrease in cardiac function in *Nedd4* icKO mice. (A) Left ventricular internal dimension in diastole (LVIDd) at baseline and 4, 22, and 50 weeks post-tamoxifen injection. (B) Left ventricular internal dimension in systole (LVIDs) at baseline and 4, 22, and 50 weeks post-tamoxifen injection. (C) Left ventricular posterior wall thickness (LVPWd) at baseline and 4, 22, and 50 weeks post-tamoxifen injection. (C) Fractional shortening (FS) at baseline and 4, 22, and50 weeks post-tamoxifen injection. (n=8-18 for all groups)

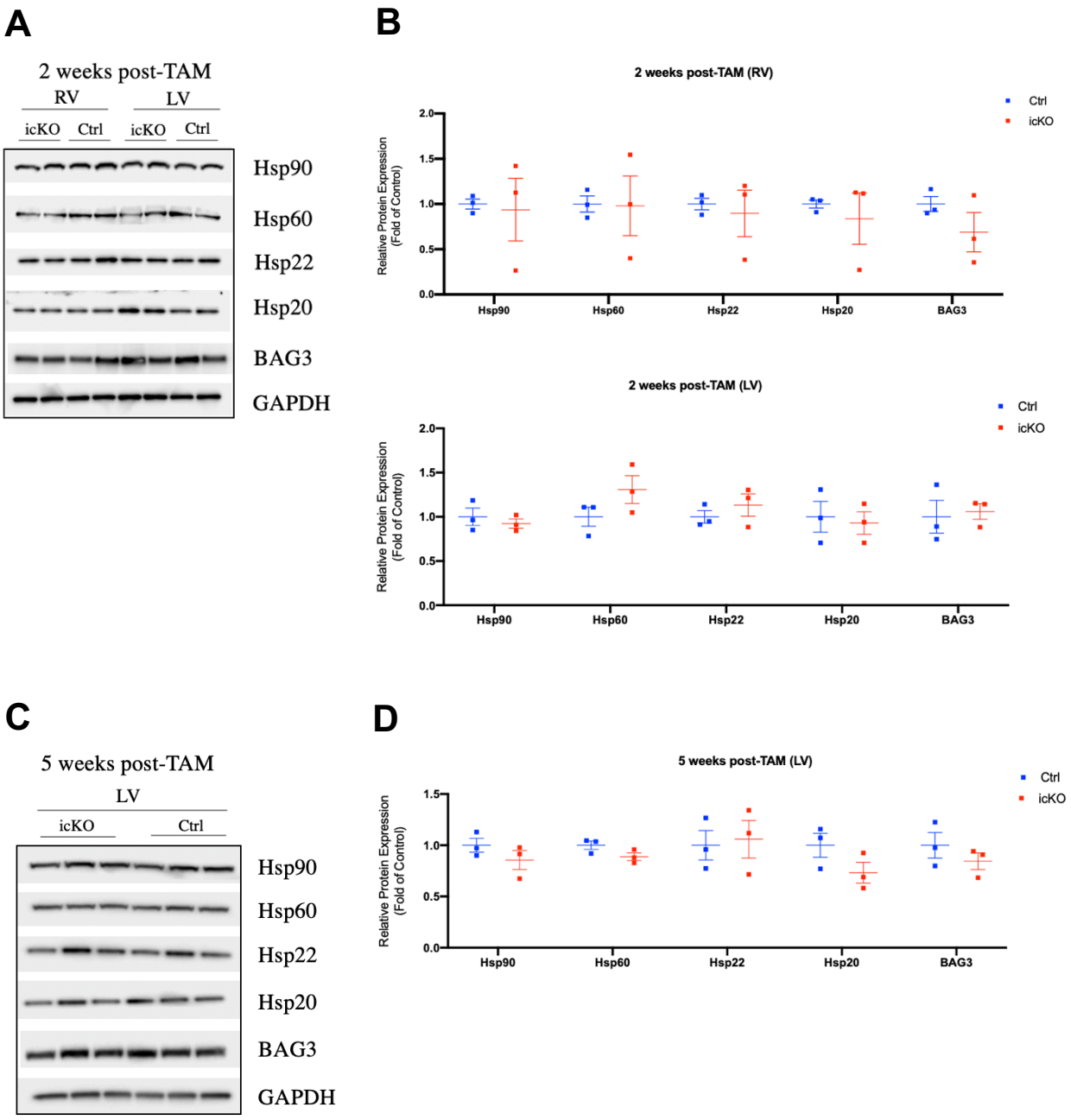

Figure 9. Deletion of NEDD4 does not result in protein stress. (A) Western blots of chaperone proteins in both right and left ventricle 2 weeks post-tamoxifen injection. (B) Quantitative analysis for right ventricle (top) and left ventricle (bottom) 2 weeks post-tamoxifen injection (n=3). (C) Western blots of chaperone proteins in the left ventricle 2 weeks post-tamoxifen injection. (D) Quantitative analysis for left ventricle 2 weeks post-tamoxifen injection (n=3).

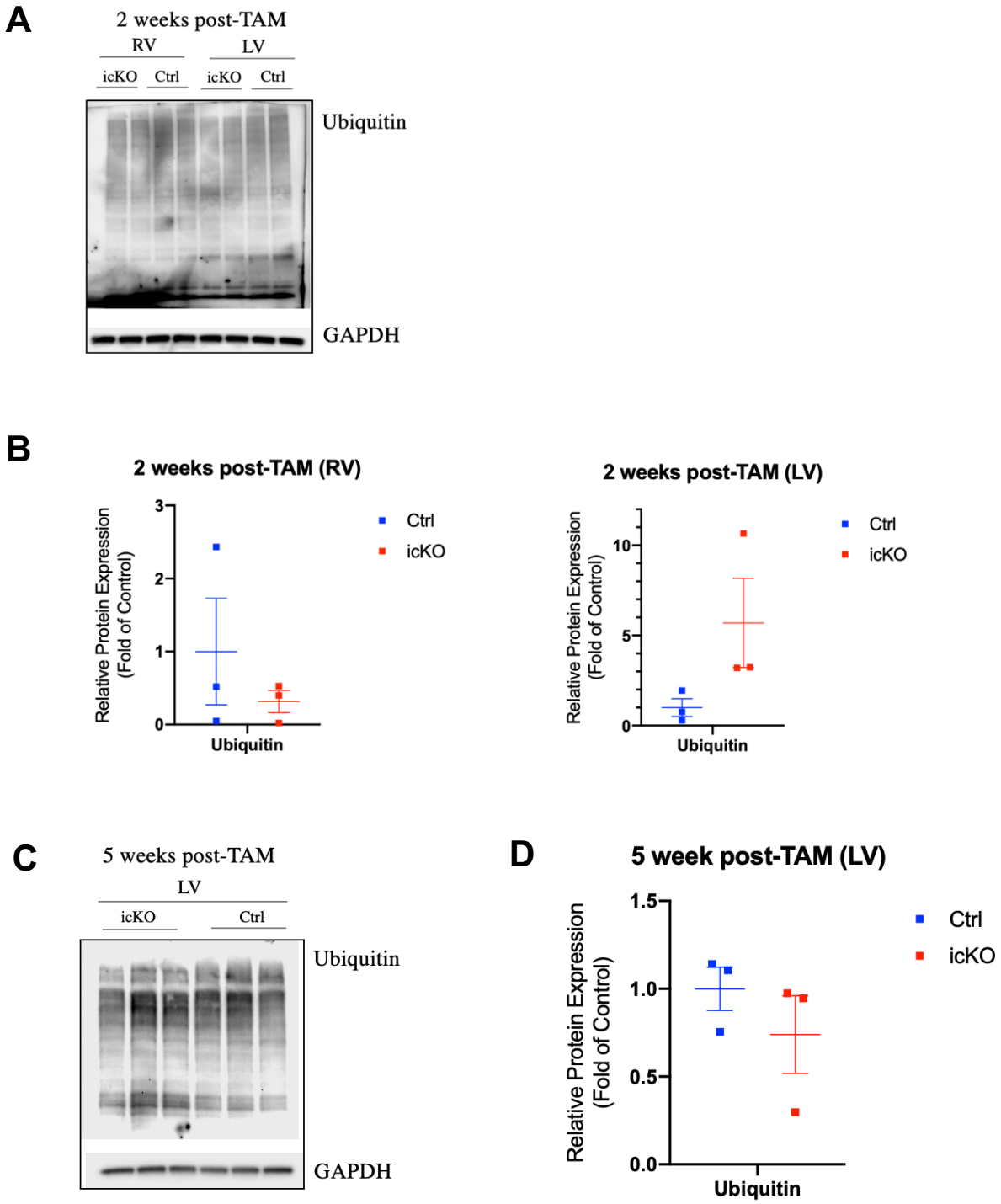

Figure 10. Deletion of NEDD4 does not result in any changes to global levels of ubiquitin. (A) Western blot of ubiquitinated proteins in right and left ventricle 2 weeks post-tamoxifen injection. (B) Quantitative analysis for right and left ventricle 2 weeks post-tamoxifen injection (n=3). (C) Western blot of ubiquitinated proteins in the left ventricle 2 weeks post-tamoxifen injection. (D) Quantitative analysis for left ventricle 2 weeks post-tamoxifen injection (n=3).

TABLES

Table 1. Genotyping Primer List

Primer	**Forward**	**Reverse**
Nedd4 flox	ACTCTTTCAGTGGACCCAACAC	TCCAAGCGTACTTACTGGGTTT
αMHC-Cre:	GCCATAGGCTACGGTGTAAAAG	ATAATCGCGAACATCTTCAGGT

Table 2. qRT-PCR Primer List

Primer	**Forward**	**Reverse**
18S	GGAAGGGCACCACCAGGAGT	TGCAGCCCCGGACATCTAAG
ANF	GATAGATGAAGGCAGGAAGCCGC	AGGATTGGAGCCCAGAGTGGACTAGG
BNP	TGTTTCTGCTTTTCCTTTATCTGTC	CTCCGACTTTTCTCTTATCAGCTC
Collagen 1a1 (Col1a1)	TCACCAAACTCAGAAGATGTAGGA	GACCAGGAGGACCAGGAAG
Collagen 3a1 (Coll3a1)	ACAGCAGTCCAACGTAGATGAAT	TCACAGATTATGTCATCGCAAAG
NEDD4 (PS1)	TACTGTTCAGGGTCCTTCCA	GGGTTTTCAGTCGGTAAAGG
NEDD4L	CACGGGTGGTGAGGAATCC	GCCGAGTCCAAGTTGTGGT
Smurf1	AGCATCAAGATCCGTCTGACA	CCAGAGCCGTCCACAACAAT
Smurf2	AAACAGTTGCTTGGGAAGTCA	TGCTCAACACAGAAGGTATGGT
Wwp1	TAAAGGTAACGGTTTCTAGTGCC	TGTGGGGTCACATTTACAATCAG

Table 2. qRT-PCR Primer List (Continued)

Primer	**Forward**	**Reverse**
Wwp2	TTTGAGAAGTCCCAGCTTACCC	CTCCAGACCTTCAGATCCAAATG
Hecw1	GACACGCCATAGGGGATAGG	CTGACGACTCAGGCTCAGC
Hecw2	AGAGCCCACACTTGTTTTAACC	TCCAAAGGTACTGGTCTCTTCAA
Itch	AACGGTGAAACGTCATGCTC	GGCCCTCTTGTTTTCCCCTG

Table 3. Antibody List

Antibody	**Source, Catalog Number**
GAPDH	Santa Cruz, sc-32233
NEDD4	ProteinTech, 21698-1-AP
Ubiquitin	Santa Cruz, sc-8017
Hsp90	Abcam, ab178854
Hsp60	Santa Cruz, sc-13966
Hsp22/HspB8	Abcam, ab151552
Hsp20/HspB6	R&D, MAB4200
BAG3	ProteinTech, 10599-1-AP