Sexually dimorphic cardiac adaptation is mediated by *Cre* expression, independent of *estrogen-receptor-α* expression

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has been approved for the Department of Molecular, Cellular, and Developmental Biology

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The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.

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Sexually dimorphic cardiac adaptation is mediated by *Cre* expression, independent of *estrogen-receptor-α* expression

Thesis Directed by Leslie A. Leinwand

Abstract

The mammalian heart is a remarkably adaptable organ. In particular, the contractile cells of the heart, the cardiac myocytes can respond to dramatic changes in metabolic and functional demand. Both clinical data and murine genetic studies suggest fundamental differences in male and female cardiac biology, including at the cellular level of the myocyte. In this thesis, I address the clinical question of why cardiovascular disease differs in males and females at the cardiac myocyte level. Specifically, I elucidate the importance and mechanism of estrogen signaling in male and female cardiac myocytes. Upon identifying *Estrogen Receptor-α* (*ERα*) as the predominant estrogen receptor in cardiac myocytes, I generated a cardiac myocyte-specific *ERα* knockout mouse using an established cardiac myocyte-specific *Cre* recombinase driver. I characterized the molecular and functional consequences of deleting *ERα* in cardiac myocytes and in doing so uncovered a novel, cardiotoxic effect of *Cre* recombinase expression in cardiac myocytes. I therefore performed a molecular, functional, and bioinformatic analysis of the most commonly used cardiac myocyte-specific *Cre* expression model. In parallel with my *in vivo* experiments, I assessed subcellular localization, signaling activation, and transcriptional competency of full-length *ERα* and two alternate spliceforms in isolated cardiac myocytes to address the cellular mechanism of *ERα* activity in cardiac myocytes. Altogether, this thesis sheds light on the sexually dimorphic nature of cardiac myocyte biology and the influence of such on overall cardiovascular function.

Dedication

This thesis is dedicated to my family, whose unconditional support made this once unthinkable accomplishment possible. In particular, to my grandmother, Charlotte Kirshner, an incredible inspiration to all who know her, but especially to me. To my parents; enablers and encouragers of anything and everything. To my twin; my very own communications specialist. Also to Margaret Thatcher (Maggie), the best and worst emotional support dog.

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Chapter 1

Introduction

1.1 Gender-specific cardiovascular biology and disease

To maintain homeostasis, the heart must constantly adapt to changes in functional demand. Exquisitely evolved to respond to both short-term and long-term stressors, the heart displays a remarkable capacity to remodel at both the molecular and functional level. Nevertheless, cardiovascular disease remains the leading cause of death in American men and women, accounting for nearly 800,000 U.S. deaths and 17.3 million deaths globally in 2015 (Mozaffarian et al., 2015). With nearly one in every three American deaths being attributable to cardiovascular disease, there is a profound need for an improved understanding of the basic biology of the heart as well as the processes and molecular players that facilitate pathological remodeling.

Although cardiac disease afflicts both men and women, sexual dimorphisms exist with regard to incidence, symptoms, clinical outcomes, and mortality rates. Notably, the incidence and mortality rate of cardiovascular disease is equivalent in prepubescent males and females but higher in males following the arrival of sex hormones (Mozaffarian et al., 2015). When sex hormones are lost following the onset of menopause, women lose their apparent cardioprotection and begin to suffer from cardiovascular disease disproportionately to males (McKee et al., 1971; Mendelsohn and Karas, 2005). This observational correlation between sex hormone status and sexually dimorphic cardiac protection led biologists and clinicians to postulate a pivotal role for sex hormones in the heart.

Decades of basic biological research have helped delineate the importance of sex

hormone signaling in the heart. The female sex hormone, 17β-estradiol or estrogen (E2) has, in particular, emerged as a powerful regulator of multiple organs and biological processes including the adult heart. E2 exacts wide-ranging effects on the reproductive organs, nervous system, metabolic network, and cardiovascular system (Gillies and McArthur, 2010; Lubahn et al., 1993; Ohlsson et al., 2000). Estrogen has also been demonstrated to signal in a multi-faceted and complex manner. In light of the intricate mechanisms with which E2 exerts both cellular and systemic effects, a precise role for E2 in the heart remains to be completely resolved.

1.2 Cardiac myocyte biology

The functional load on the adult heart is substantial. Beating more than 2.5 billion times over the lifetime of an average adult, the mammalian heart must process multiple types of biological and chemical inputs to satisfy the precise energetic needs of the body at a given time. Multiple cell types work in tight coordination during each contractile cycle to successfully move blood through the four cardiac chambers and beyond to each organ and cell in the body. Although the contractile cells of the heart, the cardiac myocytes, are the functionally relevant cell type for contraction, they are supported and dependent on high numbers of resident cardiac fibroblasts, endothelial cells, smooth muscle cells, and Purkinje fibers (Reviewed in (Kamo et al., 2015). Myocytes in the left ventricle of the heart perform the majority of force production required for hemodynamic movement during contraction. All of the myocyte studies in this dissertation will therefore utilize the left ventricular population.

Cardiac myocytes primarily remain in a terminally differentiated state postnatally (Bergmann et al., 2009; Li et al., 1996). When cardiac mass increases to accommodate

elevated functional demand, instead of dividing cardiac myocytes adapt by growing larger in a process known as cellular hypertrophy (Zak, 1974). The regulation and dysregulation of myocyte hypertrophy therefore directly influence the heart's capacity to adapt and remodel so a thorough understanding of the relevant molecular regulators is critical for effectively managing cardiac disease.

Although they make up nearly 80% of total cardiac mass, by cell number cardiac myocytes account for only 30-50% of total cardiac cells (Banerjee et al., 2007). Their relatively large size and mass is partially attributable to the expansive sarcomeric structures housed in each myocyte as well as the high numbers of mitochondria fueling the extreme energetic needs of each cell (Russell et al., 2000; Vega et al., 2015). The sarcomere is an impressive structure consisting of thin actin filaments on which thick filaments of myosin form transient sliding interactions (Reviewed in (Harvey and Leinwand, 2011)). More than 20 other proteins comprise the cardiac sarcomere and coordinated shortening of the entire structure produces myocyte contraction. Many of these sarcomeric proteins integrate ion signaling to carefully and dynamically regulate sarcomeric contraction.

Calcium signaling is required for contraction and therefore calcium flux within the sarcomere is a primary regulator of sarcomeric contraction. When calcium is bound to the sarcomeric protein, troponin C, tropomyosin-covered myosin binding sites are revealed in actin, allowing myosin and actin to interact (Lehman et al., 1994). Calcium ions move back and forth between the sarcolemma, sarcoplasmic reticulum (SR), and sarcomere between each contraction cycle in a process controlled by action potentials propagated by invaginations of the plasma membrane, or sarcolemma, known as T (transverse)-tubules

(Fabiato, 1983; Hatano et al., 2012). Calcium signaling is also involved in intracellular signaling and regulation of gene expression in cardiac myocytes. Dysregulation of calcium handling at any step in these complicated processes often negatively affects the overall health of the cardiac myocyte (Frey et al., 2000).

Regulation of cardiac myocyte hypertrophy is governed by a number of transcriptional and protein signaling events (Reviewed in (Bernardo et al., 2010)). However, two master pathways exert great control over physiological and pathological remodeling in the myocyte. The Insulin-like Growth Factor-1/Phosphoinositide 3-kinase/Protein Kinase B (IGF-1/PI3K/Akt) pathway primarily promotes physiological myocyte growth and is generally protective in the setting of cardiac disease (Matsui et al., 2002; Matsui et al., 2001; McMullen et al., 2004). In contrast, activation of the Mitogen Activated Protein Kinase (MAPK) family is generally, though not exclusively associated with pathological remodeling of cardiac myocytes (Clerk and Sugden, 2006; Yamazaki et al., 1993). Crosstalk between these and other important myocyte signaling pathways forms complicated networks with multiple levels of control over cellular hypertrophy and contractile dynamics.

1.3 Estrogen Receptor signaling

Of particular relevance to sex-specific cardiac myocyte biology is estrogenic signaling. E2 binds to two distinct nuclear receptors, estrogen receptor-α (ERα) and estrogen receptor-β (ERβ). Upon binding, the ligand/receptor pair dimerizes and binds gene promoters containing estrogen response elements (EREs) where it induces gene expression changes (Beato, 1989). This classical, nuclear E2 signaling mechanism is complemented by an alternative cytosolic signaling mechanism in which E2-ER may

bind additional signaling molecules outside of the nucleus such as the Akt (protein kinase B) signaling molecule, PI3K (Simoncini et al., 2000). ERs may also be activated in an estrogen-independent manner in response to various types of growth factor signaling including Epidermal Growth Factor (EGF), Mitogen Activated Protein Kinase (MAPK) or PI3K signaling (El-Tanani and Green, 1997; Hall et al., 2001). Many of these interacting E2-ER signaling components have established roles in cardiac biology, as described above (Patten et al., 2004). Conversely, the MAPK signaling pathway has also been reported to be regulated by E2-ER signaling (Geraldes et al., 2003) thus providing further evidence for a role of E2-ER signaling in hypertrophic cardiac remodeling (Figure 1.1).

While once thought to be functionally redundant, *ERα* and *ERβ* appear to have separable cellular roles and therefore warrant individual consideration in the context of cardiac hypertrophy. It has been previously shown, for example, that ERα and ERβ have distinct cellular localizations in human breast cancer cells (Chen et al., 2004). In addition, though their DNA-binding domains are highly homologous (~97%), the amino-termini have much lower homology (~60%), which likely results in differential interactions with transcriptional co-activators and gene targeting (McInerney and Katzenellenbogen, 1996). Further, *ERα* knockout (KO) and *ERβKO* mice have distinct phenotypes. For instance, *ERαKO* males have an exacerbated response to cardiac pressure overload compared to *ERαKO* females while *ERβKO* females respond with increased pathological hypertrophy compared to *ERβKO* males in the same pressure overload model (Skavdahl et al., 2005). The differences between the two receptors in cellular localization, amino-acid sequence, and total KO phenotype support the hypothesis that each receptor has a distinct function

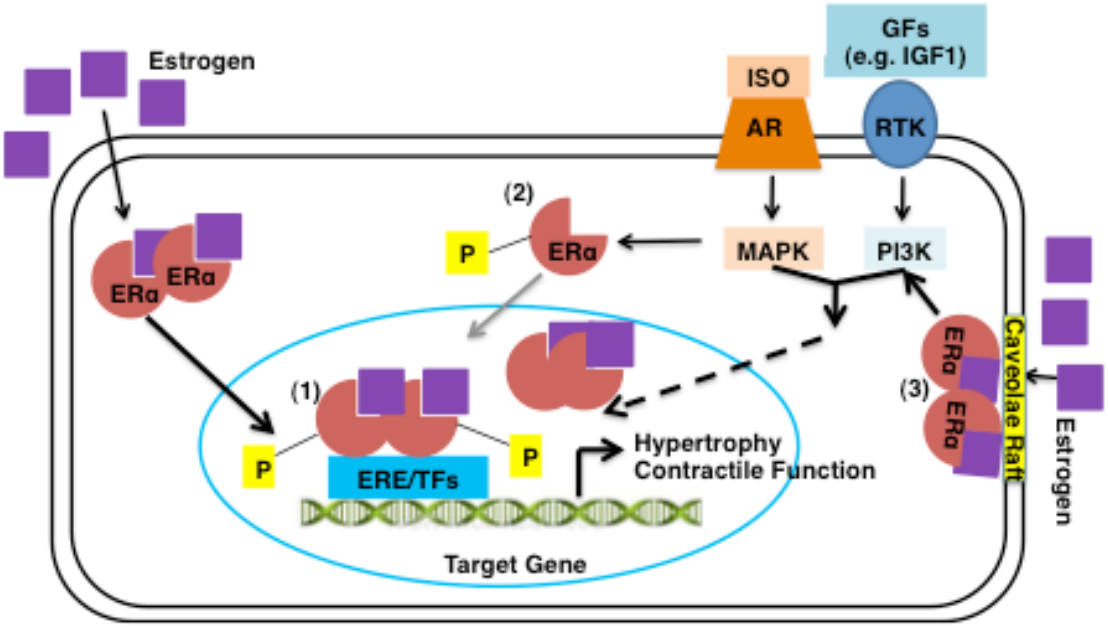

Figure 1.1: Potential mechanism for ERα regulation of cardiac myocyte biology. ERα can signal as a transcription factor (1), in a ligand independent manner through activation by kinases like MAPK and PI3K (2) or by activation of rapid signaling in the cytoplasm (3). RTK: Receptor Tyrosine Kinase, AR: Adrenergic Receptor, ERE: Estrogen Response Element, TF: Interacting Transcription Factor, P: Phosphorylation event.

within the myocardium.

The reported *ERKO* phenotypes are strongly supportive of an important role for ER signaling in the cardiac myocyte population but ER expression and requirement in other important cell types in the heart convolutes a definitive role for ERs in the myocytes themselves. *ERα* is critical, for example in protecting against pathological cardiac remodeling induced by vascular injury, suggesting *ERα* importance in resident smooth muscle and endothelial cells (Pare et al., 2002). In contrast, in the cardiac fibroblast population, *ERβ* plays a critical role in regulating cardiac fibrosis stimulated by Angiotensin II and Endothelin-1 treatment (Pedram et al., 2010). These established and essential roles for ERs in non-myocyte cell types during cardiac remodeling make difficult the interpretation of *ERKO* phenotypes with respect to the cardiac myocyte

population.

1.4 Transgenic mouse models for studying cardiac myocyte biology

Cardiac remodeling is routinely studied using *in vitro* and *in vivo* mammalian models. Such models have uncovered hundreds of regulators of molecular and functional remodeling and their specific roles in these processes. Of particular importance to understanding the requirement for specific genes and their encoded proteins in biological processes is the use of tissue and cell-specific gene targeting. *In vitro,* genes may be knocked down using RNA interference (RNAi) technology or siRNA gene targeting. It has been recently shown, however, that genetic knockdown experiments fail to phenocopy analogous genetic deletion (Rossi et al., 2015). Further, the heart relies on multi-cell type interactions to maintain function, especially to respond to adaptive stimuli (Kamo et al., 2015). Thus, *in vivo* genetic deletion remains the gold standard for identifying gene function and requirement.

In light of the fact that the heart is a complex mixture of cell types, gene deletion must be targeted to a particular cell population for the study of genes involved in more than one cell type. For the myocyte population, specific gene deletion is often achieved using cardiac myocyte-specific recombinase expression in combination with a transgenic mouse engineered with recombination sites flanking a gene of interest (Reviewed in (Davis et al., 2012)). Several cardiac myocyte-specific promoters allow for myocyte-specific recombinase expression. Among them, the *Nkx2.5* (Reecy et al., 1999) and *CTnT* (Wang et al., 2001) promoters are popular and effective for driving recombination in developing heart while the *αMyHC* (Agah et al., 1997; Subramaniam et al., 1991) promoter is most often used and highly efficient at driving recombination in adult

myocardium (Appendix II).

Another approach to targeted gene recombination in cardiac myocytes is using a temporally restricted, inducible recombinase. This strategy allows for the study of sudden gene loss at a specific age or time point during disease. The most commonly utilized iteration of this approach harnesses the power of a mutant version of the ligand binding domain of *ER* (MER) fused twice to the commonly used *Cre* recombinase (Verrou et al., 1999). Although relatively insensitive to endogenous E2, the MerCreMer fusion protein is highly sensitive to tamoxifen, a synthetic E2 analog (Feil et al., 1997). Thus, in conjunction with a cardiac myocyte-specific promoter, transient treatment with tamoxifen allows for temporally restricted *Cre* expression and recombination. Since myocytes remain mostly post-mitotic, a single course of tamoxifen should theoretically induce recombination in most myocytes for the remainder of a *MerCreMer* mouse's life. Such an approach has been successfully utilized using the *αMyHC* promoter driving *MerCreMer* expression (Sohal et al., 2001).

Recombination efficiency varies among *Cre* promoters, whether inducible or constitutive (Reviewed in (Davis et al., 2012)). Locus accessibility can also directly influence *Cre* recombinase efficiency. For the most commonly used developmental promoters, reported efficiency of recombination ranges from 90-100% (Kwon et al., 2007; Maillet et al., 2010; McFadden et al., 2005) while *Cre* expression driven by the *αMyHC* promoter has reported efficiency of 70-80% of myocytes by three weeks of age (Agah et al., 1997; Oka et al., 2006). A less commonly used *αMyHC* promoter *Cre* line was also generated by Dale Abel and colleagues and was demonstrated to induce nearly 100% recombination efficiency at the *Glut4* locus (Abel et al., 1999). Inducible *Cre*

expression also drives effective recombination in cardiac myocytes. The *αMyHC-MerCreMer* line has reported recombination efficiency of >80% following four doses of tamoxifen (Sohal et al., 2001) and high efficiency of recombination was also observed with just a single dose of tamoxifen (Lexow et al., 2013). Thus, with a readily accessible locus, cardiac myocyte-specific *Cre* recombination is robust when *Cre* expression is driven by a variety of different promoters.

Although cardiac myocyte-specific *Cre* lines are commonly and effectively used to study cardiac myocyte-specific genetic function, there are several caveats and considerations that should be noted. Many of the transgenic cardiac myocyte-specific *Cre* expression lines have an unknown number of transgene insertions that can lead to non-physiological promoter activity and excessive Cre protein. Similarly, failure to determine the insertion site for any given *Cre* transgene presents the possibility that an endogenous gene locus has been disrupted. Disruption of an endogenous gene by the *Cre* transgene can convolute interpretation of cardiac myocyte phenotype attributed to targeted deletion of a floxed gene of interest. Finally, inducible *Cre* expression lines must take into account the potential for cardiotoxic effects of the chemical inducing agent. Tamoxifen, for example, has well-known cardiotoxic effects, especially in conjunction with *Cre* expression (Bersell et al., 2013; Koitabashi et al., 2009). Thus, while targeted expression of *Cre* in cardiac myocytes remains a powerful tool for assessing myocyte-specific gene function, use of these tools must include appropriate controls and measures to minimize off-target effects.

1.5 Questions addressed in this study

In this thesis I will present data that helps clarify the role of E2-ER signaling in cardiac myocytes. In Chapter 2 I have assessed the expression pattern, localization, and signaling capacity of cardiac myocyte-relevant ERs in isolated cardiac myocytes. In Chapter 3 I determined the requirement for the predominant cardiac myocyte ER, *ERα*, *in vivo* by generating a cardiac myocyte-specific *ERα* knockout mouse using an *ERα* floxed mouse and the widely used *αMyHC-Cre* transgenic mouse line (Agah et al., 1997). I then measured the consequences of cardiac myocyte *ERα* deletion at the molecular, histological, functional level in male and female mice. Although only minor differences were observed at the molecular level in young, female *ERα* cardiac myocyte null mice, substantial functional differences were observed in both male and female aging *ERα* cardiac myocyte null mice. Although initially suggestive of a critical role for *ERα* in lifetime cardiac maintenance, based on anecdotal evidence of *αMyHC*-Cre cardiotoxicity I hypothesized that the phenotype I observed might be attributable to myocardial *Cre* expression. I therefore characterized the effect of myocardial Cre expression using the *αMyHC-Cre* transgene using a combined molecular, functional, and bioinformatics approach in Chapter 4 (Pugach et al., 2015). Together these findings help establish a more accurate role for cardiac-myocyte *ERα* and shed important light on the pitfalls of transgenic mouse modeling.

Chapter 2

Estrogen-Receptor-α signaling in cardiac myocytes

Abstract

Mounting evidence suggests that estrogen signaling plays a critical role in the heart. Although a role for estrogen regulation of cardiac biology is hypothesized, the precise requirement for estrogen signaling is complicated by the complex signaling capacities of estrogen and its likely role in many, if not all of the diverse cell types of the mammalian heart. To more carefully understand the role and mechanism of estrogen signaling in cardiac myocytes, the contractile cells of the heart, I studied the expression, localization, transcriptional activity, and signaling activity of estrogen-receptors in isolated cardiac myocytes. I found Estrogen Receptor (ER) expression restricted to *Estrogen Receptor-α* (*ERα*) in cardiac myocytes. Full length *ERα* primarily localizes to nuclei in cardiac myocytes where it is competent to activate transcription. Truncated, alternate isoforms of *ERα* have differential transcriptional activity in cardiac myocytes and primarily localize to nuclei. Neither full length nor truncated *ERα* isoforms are competent to activate MAPK or PI3K signaling in cardiac myocytes. Together these data support a role for *ERα* at the level of transcription in cardiac myocytes.

2.1 Introduction

Decades of research have shed light on the mechanism of ERα's cellular influence. In several non-myocyte populations, it has been demonstrated that ERα may signal in a variety of ways. The classical, genomic mechanism of estrogen signaling involves ligand-dependent DNA or transcription factor binding and subsequent regulation of transcription

(Yamamoto, 1985). Palindromic hormone response elements in DNA called estrogen response elements (EREs, AGGTCAnnnTGACCT) provide an optimal recognition sequence for ER-E2 dimer binding (Berg, 1989), although transcription regulation can also occur through interaction with other transcription factors or ER recognition of variations to the consensus ERE sequence (Batistuzzo de Medeiros et al., 1997; Galien and Garcia, 1997). Nongenomic mechanisms of estrogenic action have been more recently described (Reviewed in (Farach-Carson and Davis, 2003)). These estrogen-initiated signaling events occur on the order of seconds or minutes and are considered much too rapid to attribute to traditional genomic signaling mechanisms. Thus, E2-ER action can occur through two distinct mechanisms.

Similar to other nuclear hormone receptor genes, the human *ERα* locus is complex and undergoes complicated alternative splicing and promoter usage (Flouriot et al., 1998; Kastner et al., 1990). The *ERα* locus encodes a 66 kDa protein with six distinct domains, A-F (Figure 2.0) (Greene et al., 1986). The NH_2-terminal (N-terminal) A/B domains encompass a ligand-independent transcription activation domain termed AF1 which interacts with co-activators while the COOH-terminal (C-terminal) AF2 domain codes for a ligand-dependent transactivation domain (Beato et al., 1995; Kumar and Thompson, 1999). *ERα*'s C and D domains encompass the DNA-binding domain and dimerization domain of the protein, respectively while domain E encodes the ligand-binding portion of *ERα* (Figure 2.1) (Kumar and Thompson, 1999).

Several variations of full length *ERα* have been reported. A 46 kDa N-terminal truncation of full length *ERα* was first identified in human MCF7 breast cancer cells (Flouriot et al., 2000). *ERα46* is transcribed from an alternative promoter and lacks the

AF-1 transactivation domain of full length *ERα66* but is otherwise identical (Figure 2.1). *ERα46* expression has been observed in endothelial cells, ovary, lung and kidney (Flouriot et al., 2000; Li et al., 2003). Interestingly, a 46 kDa band was also identified in the membrane fraction of adult cardiac myocyte lysates using an ERα antibody (Ropero et al., 2006) suggesting a potential role for this ERα variant in cardiac myocytes.

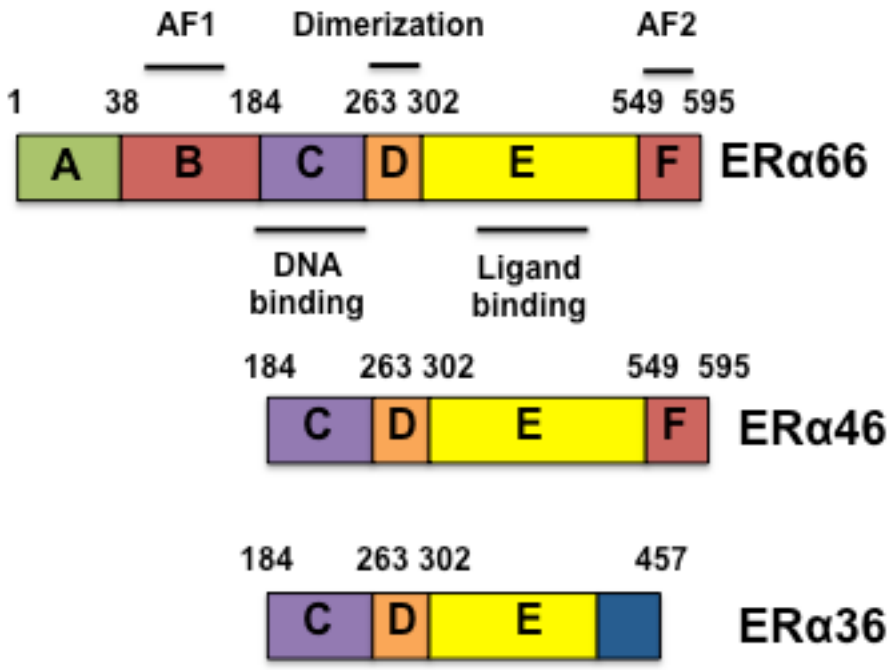

Figure 2.1: *ERa* structure and splice variants. Full length *ERa* is 66 kDa and includes 2 transactivation domains, AF1 (A/B) and AF2 (F), a ligand binding domain (C), a DNA binding/dimerization domain (D) and a ligand binding domain (E). *ERa46* and *ERa36* are N-terminal truncations that lack the AF-1 domain. *ERa36* includes a unique C-terminus.

Microscopic and biochemical analyses have localized the *ERα46* splice variant to the plasma membrane and cytosol of cell types in which it has been identified (Li et al., 2003; Ropero et al., 2006) although it is also competent to activate transcription (Figtree et al., 2003). A single report suggests colocalization of cardiac myocyte membrane ERα46 with α-actinin at T-tubular membranes using immunofluorescence of rat cardiac

myocytes (Ropero et al., 2006). Similarly immunofluorescence was used to localize ERα to myocyte sarcolemma and intercalated discs in human cardiac myocytes (Mahmoodzadeh et al., 2006). Although these data are suggestive of a role for ERα46 in regulating myocyte contraction dynamics or structure, these findings remain to be recapitulated. Consistent with its localization at the membrane or in the cytosol, ERα46 has been reported to induce rapid, non-genomic signaling in human breast cancer cells and endothelial cells (Li et al., 2003; Marquez and Pietras, 2001). Whether ERα46 plays a similar role in cardiac myocytes remains to be determined.

A more recently identified human *ERa* variant, *ERa36* is another N-terminal truncation lacking the A/B AF1 domain. *ERa36* also lacks the C-terminal activation domain of full length and ERα46 and instead contains a unique C-terminal sequence (Wang et al., 2005). *ERa36* is transcribed from a promoter located in the first intron of *ERa* and its expression has been observed in multiple cell and tissue types including several breast cancer cells lines and a number of different mouse tissues (Irsik et al., 2013; Wang et al., 2005; Zheng et al., 2010)**.** When overexpressed in HEK293 (Human Embryonic Kidney) cells or MCF7 breast cancer cells the 36 kDa *ERa* isoform has been shown to regulate rapid signaling pathways like the pERK/MAPK pathway in conjunction with either estrogen treatment or addition of an *ERa36* specific agonist (Kang et al., 2010)**.** The demonstrated ability of cardiac myocytes to also respond rapidly to estrogen treatment through activation of analogous pERK/MAPK signaling (Nuedling et al., 1999b) and the importance of this pathway in regulating cardiac myocyte biology (see Chapter 1) call for a more thorough investigation of the ability of specific *ERa* isoforms to regulate these pathways in cardiac myocytes.

As described, when bound by E2 both full length ER and alternate isoforms of ER can function both as nuclear transcription factors and cytoplasmic signaling activators. Further, ERα and ERβ have been shown to differentially localize depending on cell type or stimulus (Nuedling et al., 1999a; Ropero et al., 2006). Live-cell and/or antibody-independent imaging of ER localization in cardiomyocytes have not yet been reported and may provide clues to ER function in cardiac myocytes. Therefore, I examined the nuclear, cytoplasmic, and membrane distribution of the ERs and the contribution of estrogen signaling in either cellular compartment. These studies help reveal the cellular location from which important downstream signaling events originate in cardiac myocytes and may inform upon more targeted cardiac myocyte-relevant ER therapeutics.

2.2 Materials and methods

Cardiac myocyte isolation

Neonatal rat ventricular myocytes (NRVMs) were isolated from 1 day old pups as previously described (Maass and Buvoli, 2007). Briefly, hearts were harvested, atria removed, and ventricles digested with trypsin. Fibroblasts were removed by preplating the trypsin-digested cell preparations. Adult rat ventricular myocytes (ARVMs) were isolated from ~300g rats as previously described (Haines et al., 2012b). Briefly, hearts were harvested then digested with collagenase using a Langendorff perfusion apparatus. Following dissection of the left ventricle, myocytes were enriched using mesh filtration and successive centrifugation in increasing amounts of calcium solution. Cells were serum starved for at least 24 hours prior to all assays requiring estrogen treatment.

Gene expression

Total RNA was purified using TRI Reagent (Ambion) according to the

manufacturer's protocol. cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen) and random hexamer primers. Gene expression was determined by qRT-PCR using SYBR Green dye and gene specific primer sets (Appendix I) and a Bio-Rad CFX-96 Real-Time PCR system.

ERa overexpression studies

For preliminary studies of *ERα* and *ERβ* localization, the human *ERα* or *ERβ* open reading frame was cloned into pEGFP:C1 using SalI and BamHI restriction sites. For mCherry-ERβ, mCherry cDNA sequence was substituted for EGFP sequence using NheI and BspEI restriction sites. For subsequent studies, either the full length human *ERα66*, *ERα46,* or *ERα36* cDNA open reading frame was cloned into pEGFP:N1 using EcoRI and BamHI restriction sites. For N-terminally tagged constructs, a flexible linker (TCCGGAGCCGGCGCTGGTGCTGGTGCTGGCGCCATC) was placed between the fluorophore and *ERα* sequence. For C-terminally tagged constructs a similar flexible linker was also placed between the EGFP and *ERα* sequence (CCACCGGTCGCCACCATG). NRVM transfection was achieved using Rat Cardiomyocyte-Neonatal Nucleofector Kit (Lonza) with 2 μg of Endotoxin Free DNA according to the manufacturer's protocol. H2B-BFP was a kind gift from the Voeltz Lab (CU Boulder).

Subcellular fractionation

Cells were fractionated according to manufacturer protocol (NEB #9038). Following fractionation, lysates were sonicated in a water bath, boiled, and centrifuged. Fractions were then analyzed by western blot as follows. 15 μL of lysate was loaded onto a 10% SDS-PAGE gel. Fractionation was confirmed using the following antibodies:

Histone 3 (Cell Signaling 4499s): Nuclear fraction, Caveolin-3 (Santa Cruz 5310): Membrane fraction, and Gapdh (Cell Signaling 2118): Cytoplasmic fraction. EGFP-tagged ERα was then detected using anti-GFP (Santa Cruz 8334). Quantification of GFP in each fraction was performed using ImageJ.

Adenoviral constructs

Adenovirus production was performed using the AdEasy kit (Qbiogene) with modifications (Resnicow et al., 2010). Briefly, after cloning each construct into pShuttle-CMV, the shuttle vector was linearized with PmeI and homologously recombined with pAdEasy in bacteria. Successfully recombined plasmids were linearized with PacI and transfected into HEK293 cells stably expressing the E1 protein to complement pAdEasy for replication competence. Infected cell lysates were used to infect increasing numbers of cells, then virus isolated from the lysates by sequential step and linear CsCl gradients. Purified virus was stored at -20°C in 100 mM Tris pH 7.5, 250 mM NaCl, 1 mM MgCl2, 1 mg/ml BSA, 50% glycerol. Multiplicity of infection (MOI) for each virus was chosen such that final protein expression was comparable between *ERα* isoforms and >90% of cells were EGFP-positive for *ERα* containing adenoviruses. MOIs used for Adeno-*EGFP*-only, Adeno-*ERα36*, Adeno-*ERα46*, and Adeno-*ERα66* were 2, 0.5, 6, and 0.3 respectively.

Microscopy

Live cells were imaged using time-lapse confocal microscopy on a Nikon Eclipse TE 2000-U microscope coupled with an electron-multiplying charge-coupled device camera (Cascade II; Photometrics) and a Yokogawa spinning disc confocal system (CSU-Xm2; Nikon). Cells were plated on 1% gelatin (NRVMs) or laminin (ARVMs) coated

glass coverslips, or on 35 mm glass bottom dishes (MatTek). Fixed cells were imaged on a Nikon A1R Confocal microscope. High throughput microscopy was performed in 96-well glass bottom plates (MaTek) on a Molecular Devices ImageXpress Micro XL High-Content Screener. Cells were stained with Phalloidin-texas red and nuclei were marked with Hoechst dye. EGFP-ERα was then co-localized with either marker.

Reporter assays (ERE-luciferase)

24-hour serum-starved NRVMs plated in 6-well dishes were infected with *ERα-EGFP* adenoviruses or control, *EGFP*-only adenovirus along with *ERE-luciferase and* control *β-galactosidase* containing adenovirus. 4 hours after infection, cells were treated with either vehicle or 1 nM estrogen. 12 hours after hormone treatment (16 hours post infection), cells were lysed in 200 µL of Reporter Lysis Buffer (Promega E3971). Luciferase activity was quantified using 50 µL LARI substrate (Promega E1500) and 10 µL of cell lysate. Luciferase activity was normalized to β-galactosidase activity using ß-Galactosidase Enzyme Assay System (Promega E2000).

Signaling activation

24-hour serum-starved NRVMs were isolated and infected with *ERa-EGFP* adenoviruses or control, GFP-only adenovirus. 36-40 hours post-infection, *ERa-EGFP* expression was confirmed using live-cell fluorescence microscopy. Cells were treated with either vehicle or 100 pM estrogen for 5 minutes, washed in PBS, and lysed in Ripa buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP40, 0.5% Na-deoxycholate, 0.1% SDS, complete protease inhibitor cocktail (Roche) and the following phosphatase inhibitors: 1 mM PMSF, 2m M NaF, 2 mM NaPPi, 1 mM Beta-Glycerophosphate, 1 mM Na-molybdate dihydrate, and 1 mM Na-Orthovanadate). Lysates were sonicated in a water

bath, centrifuged and subjected to Bicinchoninic Acid (BCA) assay (Pierce 23225) for protein quantification. 10 μg of protein were then resolved on a 4-12% Bis-Tris SDS-PAGE gel (Life Technologies) and probed with antibodies for pAkt (Cell Signaling 9275s), Akt (Cell Signaling 9272), ppERK (Cell Signaling 9101s), ERK (Cell Signaling 9102s), and Tubulin (Sigma t7816). Quantification was performed using ImageJ.

Data and statistical analysis

Data are presented as mean ± SEM. Differences between groups were evaluated for statistical significance using Student's two-tailed t test (two groups) or one-way ANOVA (more than two groups) followed by Tukey's post-hoc test for pairwise comparisons. For comparisons between multiple treatments and groups, two-way ANOVA was performed followed by Sidak's post-hoc test. *P* values less than 0.05 were considered significant unless otherwise noted.

2.3 Results

2.3.1 ER expression in cardiac myocytes

To determine the most likely biologically relevant mediator of estrogenic action in cardiac myocytes, *estrogen receptor* (*ER*) expression was profiled in isolated cardiac myocytes. In collaboration with Christa Blenck, I measured and compared expression of *ERa* and *ERβ* mRNA in neonatal rat ventricular myocytes (NRVMs) and adult rat ventricular myocytes (ARVMs) with and without 100 pM β-estradiol (E2) treatment. *ERβ* mRNA was undetectable in both NRVMs and ARVMs (data not shown) but *ERa* was detectable in both NRVMs and ARVMs (Figure 2.2). Expression was approximately 3 fold higher in adult cardiac myocytes compared to neonatal cardiac myocytes but not different between male and female cardiac myocytes. *ERa* expression in both neonatal

and adult male and female cardiac myocytes was not changed following 24 hours of E2 treatment.

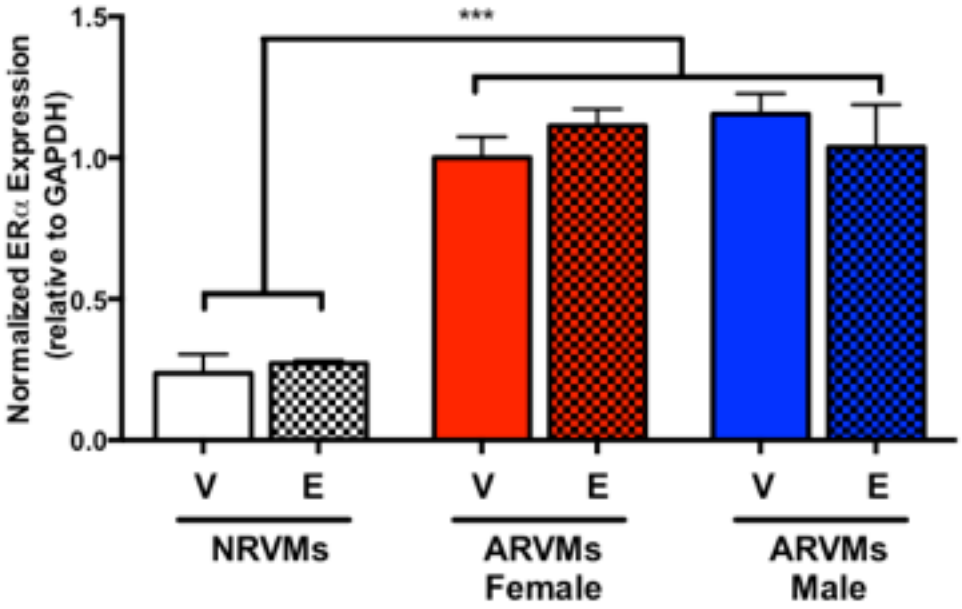

Figure 2.2: *ERa* expression in isolated cardiac myocytes. *ERa* expression in neonatal and adult rat ventricular myocytes with 24 hours vehicle (V) or 100 pM E2 treatment (E). N=3-4 animals (ARVMs) or preparations of cells/group (NRVMs), ***$P < 0.001$.

2.3.2 ERα localization in cardiac myocytes

Since antibodies for both ERs demonstrated poor specificity in my hands (data not shown), I cloned the full length open reading frames of each ER into pEGFP-C1 to study the subcellular localization of ERα and ERβ in isolated cardiac myocytes (Figure 2.3A). pEGFP-C1-ERβ was modified such that the coding sequence of *EGFP* was replaced with the coding sequence of *mCherry*. Appropriately sized full-length EGFP-ERα protein was abundantly produced in NRVMs (Figure 2.3B). Adenoviruses were also constructed using the fluorophore-tagged ER to allow for increased efficiency and uniformity of expression in NRVMs as well as studies in ARVMs which undergo extremely low transfection efficiency.

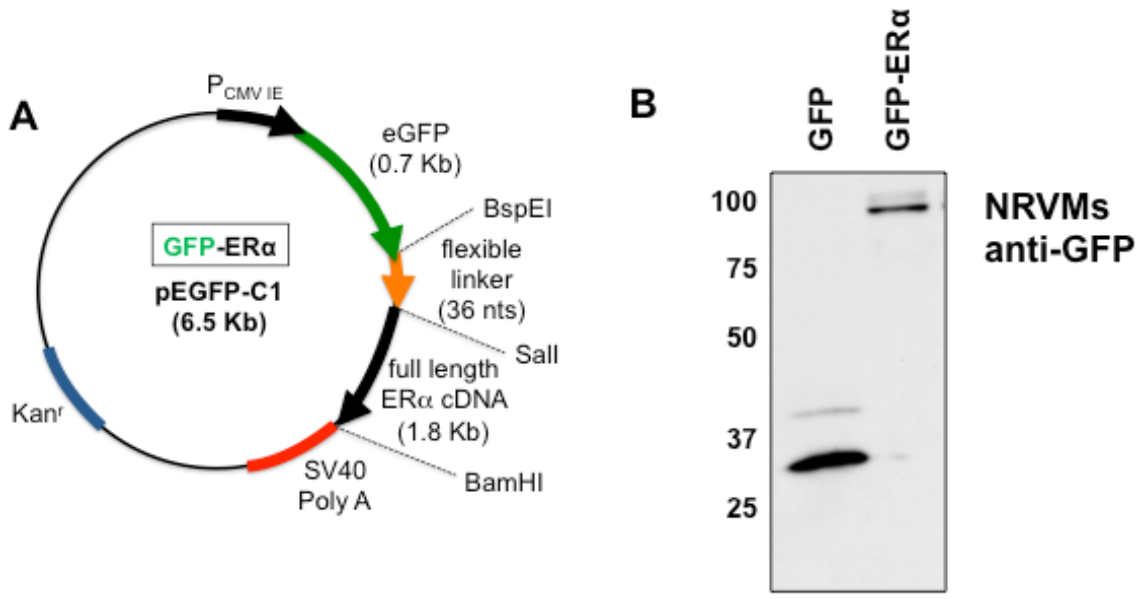

Figure 2.3: pEGFP-C1, eGFP-ERα expression construct. (A) Plasmid map for EGFP-ERα, including flexible linker restriction sites. **(B)** Appropriately sized full-length EGFP-ERα was abundantly produced in NRVMs, as detected using immunoblot with GFP antibody.

NRVMs were transfected with a nuclear marker, blue fluorescent protein-tagged Histone 2B (H2B-BFP), and either *mCherry-ERβ* or *EGFP-ERα* and imaged using time-lapse microscopy prior to and following treatment with 1 nM E2. Using live-cell confocal microscopy, both EGFP-ERα and mCherry-ERβ were found to localize primarily in myocyte nuclei, consistent with their roles as nuclear transcription factors (Figure 2.4). Localization remained mostly nuclear following 1 nM E2 treatment, although rearrangement within the nucleus was observed, suggesting chromatin redistribution (Figure 2.4). In light of the fact that ERβ was not detectable in either neonatal or adult cardiac myocytes in our hands, the remainder of my studies of ER-mediated E2 signaling focused on characterizing ERα signaling in these cells.

To study the localization of EGFP-ERα in a population of cells, high-throughput microscopy was performed. NRVMs were infected with adenovirus containing EGFP-

ERα and imaged prior to and following treatment with 1nM E2. The nuclear and cytoplasmic region of each cell was marked by Hoechst dye and phalloidin-texas red, respectively (Figure 2.5A). EGFP-ERα signal was co-localized with either nuclear or cytoplasmic regions and quantified using MetaXpress software (Figure 2.5B). Consistent with our high-magnification, confocal studies (Figure 2.4), on average 10-fold more EGFP-ERα co-localized within NRVM nuclei vs. cytoplasm regardless of E2 status (Figure 2.5).

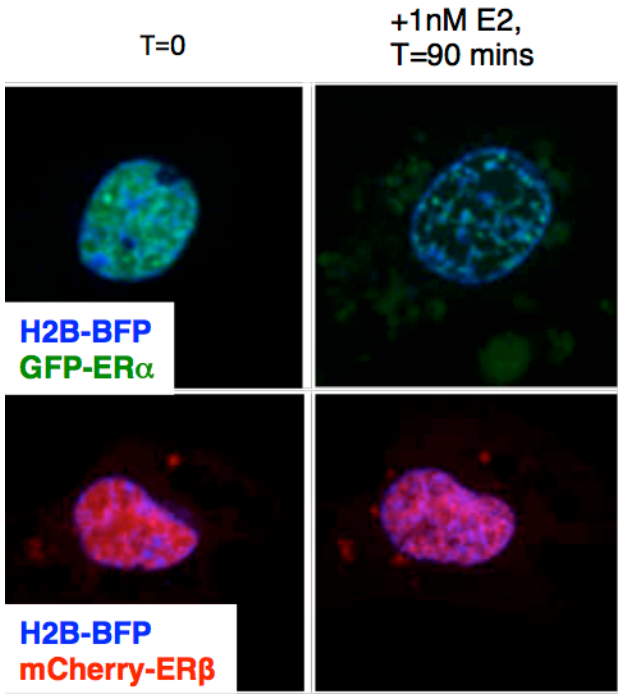

Figure 2.4: ERα and ERβ localization in NRVMs. Following overexpression by Amaxa nucleofection, ERα and ERβ predominantly localize to nuclei as evidenced by co-localization with H2B-BFP. Localization remains nuclear following 90 minutes E2 treatment.

As an additional measure of EGFP-ERα localization in NRVMs, I performed subcellular fractionation of cells followed by western blot assessment of EGFP-ERα presence in each fraction. As shown in Figure 2.6, primarily nuclear localization of EGFP-ERα was observed with <10% localization in the membrane or cytosol.

Localization became increasingly nuclear following 1 hour, but not 5 minutes, of E2 treatment. These results are consistent with my microscopy findings.

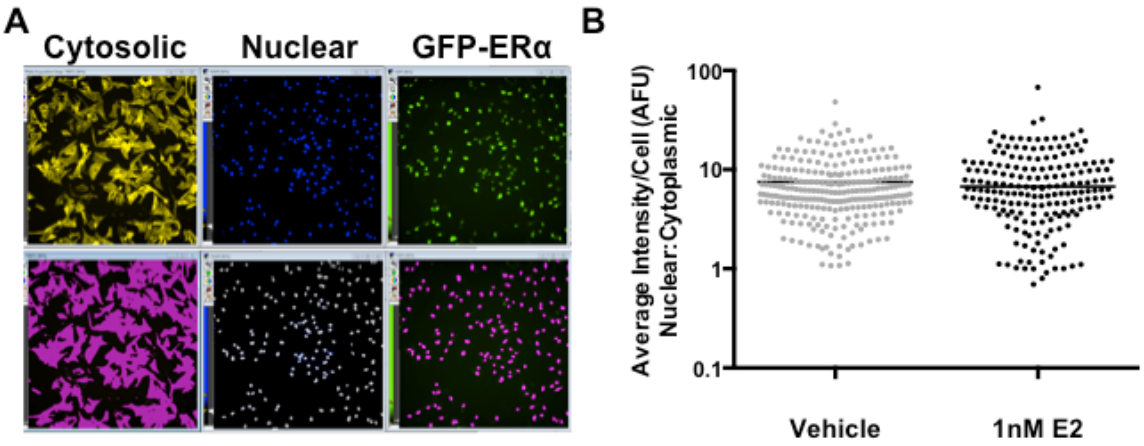

Figure 2.5: High-throughput quantification of EGFP-ERα localization in NRVMs. Following overexpression by adenoviral infection, ERα colocalization was quantified with either Hoechst dye (nuclear) or Phalloidin stain (cytoplasmic). **(A)** Representative micrographs (upper panel) and subcellular compartment masking based on staining pattern (lower panel). 20X magnification. **(B)** Quantification of EGFP-ERα based on co-localization with cytosolic and nuclear compartments in (A) +/- E2 treatment. AFU: Arbitrary fluorescence units.

2.3.3 Transcriptional activity of EGFP-ERα in NRVMs

To confirm the functional competency of the fluorophore-tagged EGFP-ERα, I assessed the ability of EGFP-ERα to activate known ERα transcriptional targets in NRVMs. EGFP-ERα was capable of activating a synthetic *estrogen-responsive element* (*ERE*) driven *luciferase* reporter (*ERE-Luciferase*). Luciferase expression was increased upon expression of EGFP-ERα and treatment with E2 (Figure 2.7A). As a positive control, *ERE-Luciferase* was measured in the *ERα*-expressing and estrogen-sensitive MCF7 breast cancer cell line. Similar results were observed for a known ER-target gene, *Pgr* (Figure 2.7B) (Petz et al., 2004). *EGFP-ERα* over-expression (OVX) increased

expression of *Pgr* compared to expression of *EGFP* alone or no overexpression. Treatment with estrogen in the presence of EGFP-ERα further enhanced *Pgr* transcription. *Pgr* expression was blocked by pretreatment with the known, high-affinity ER-antagonist, ICI 182,780 (ICI). Importantly, neither *ERE-luciferase* nor *Pgr* expression were inducible following estrogen treatment in the absence of *EGFP-ERα* overexpression. Together these results indicate that, in NRVMs, eGFP-ERα is capable of both estrogen-independent and estrogen-dependent activity. In addition, the low abundance of endogenous *ERα* renders NRVMs incapable of a classical transcriptional response to treatment with E2 in the absence of *ERα* overexpression.

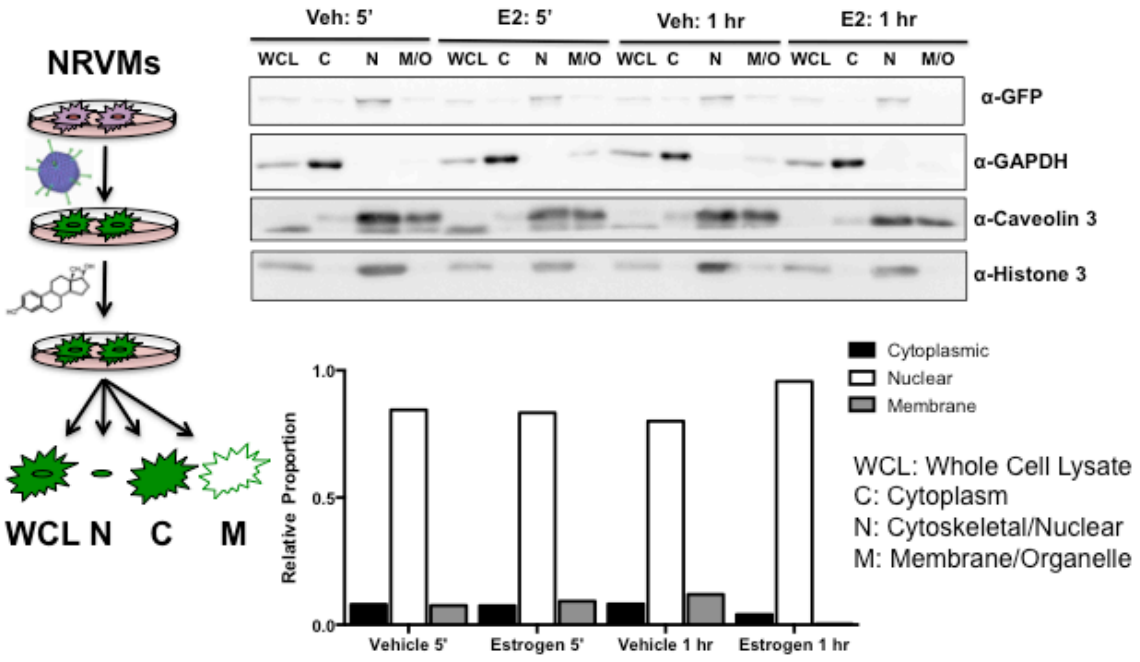

Figure 2.6: Subcellular fractionation of NRVMs and GFP-ERα localization. Schematic representation of EGFP-ERα expression, estrogen treatment, and NRVM fractionation assay. Following overexpression by adenoviral infection, GFP-ERα localization was quantified in fractionated cell lysates. Subcellular fraction identity was verified by the presence of either GAPDH (cytosol), Caveolin-3 (Membrane), or Histone-3 (nucleus).

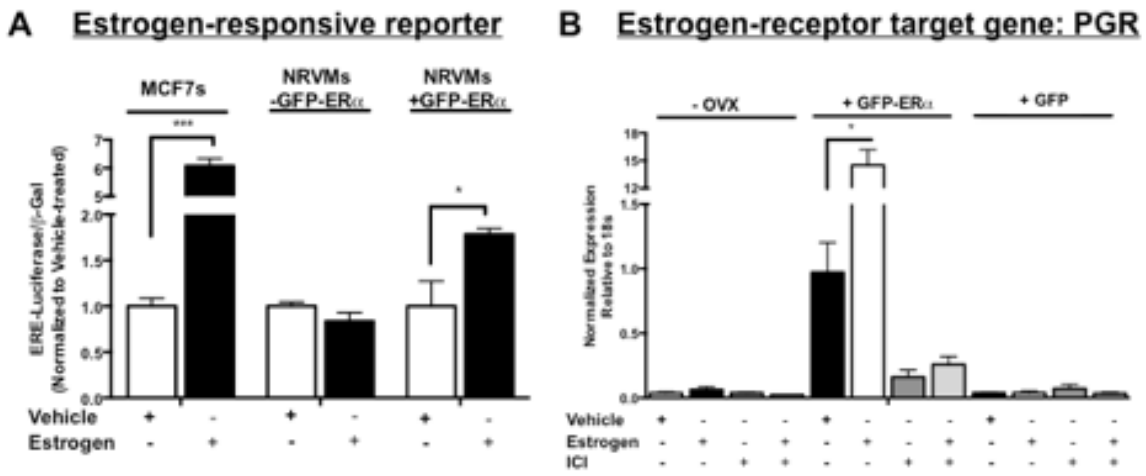

Figure 2.7: EGFP-ERα is transcriptionally competent in NRVMs. (A) Following overexpression with GFP-ERα and treatment with E2, ERE-luciferase-reporter activity was measured. MCF7 breast cancer cells: ERα-expressing cell line, positive control. **(B)** Following NRVM overexpression of GFP-ERα, GFP alone, or mock (no OVX) and treatment with 1 nM E2 or 100 nM ICI (ICI 182,780), mRNA expression of ERα target, *Pgr* was measured. **P* < 0.05.

2.3.4 Rapid signaling activity of EGFP-ERα in NRVMs

Since E2 has been shown to rapidly activate both the MAPK and PI3K signaling pathways in cardiac myocytes and other cell types (Nuedling et al., 1999b; Simoncini et al., 2000), I next asked whether EGFP-ERα was capable of rapid activation of either of these pathways in cardiac myocytes. Interestingly, Akt activation (phosphorylation of Akt at Thr308) was observed following over-expression of *EGFP-ERα*, independently of E2 or ICI status (Figure 2.8A). Although activation of Akt was not observed in control cells infected with adenovirus expression GFP alone, viral load may differ between constructs and higher EGFP-ERα relative to GFP-alone viral load may be responsible for induction of pAkt in these cells. It should be noted, however, that ligand-independent activity of ERα is well-documented (Weigel and Zhang, 1998).

As shown in Figure 2.8B, rapid activation of the MAPK pathway, as evidenced by phosphorylation of ERK1/2 (Thr202/Tyr204), was not observed except following

treatment with known agonist, EGF (Pierce et al., 2001). Thus, although NRVMs are capable of rapid activation of MAPK signaling, neither treatment with E2 nor overexpression of ERα alone or in combination with E2 treatment were competent to activate MAPK signaling in NRVMs.

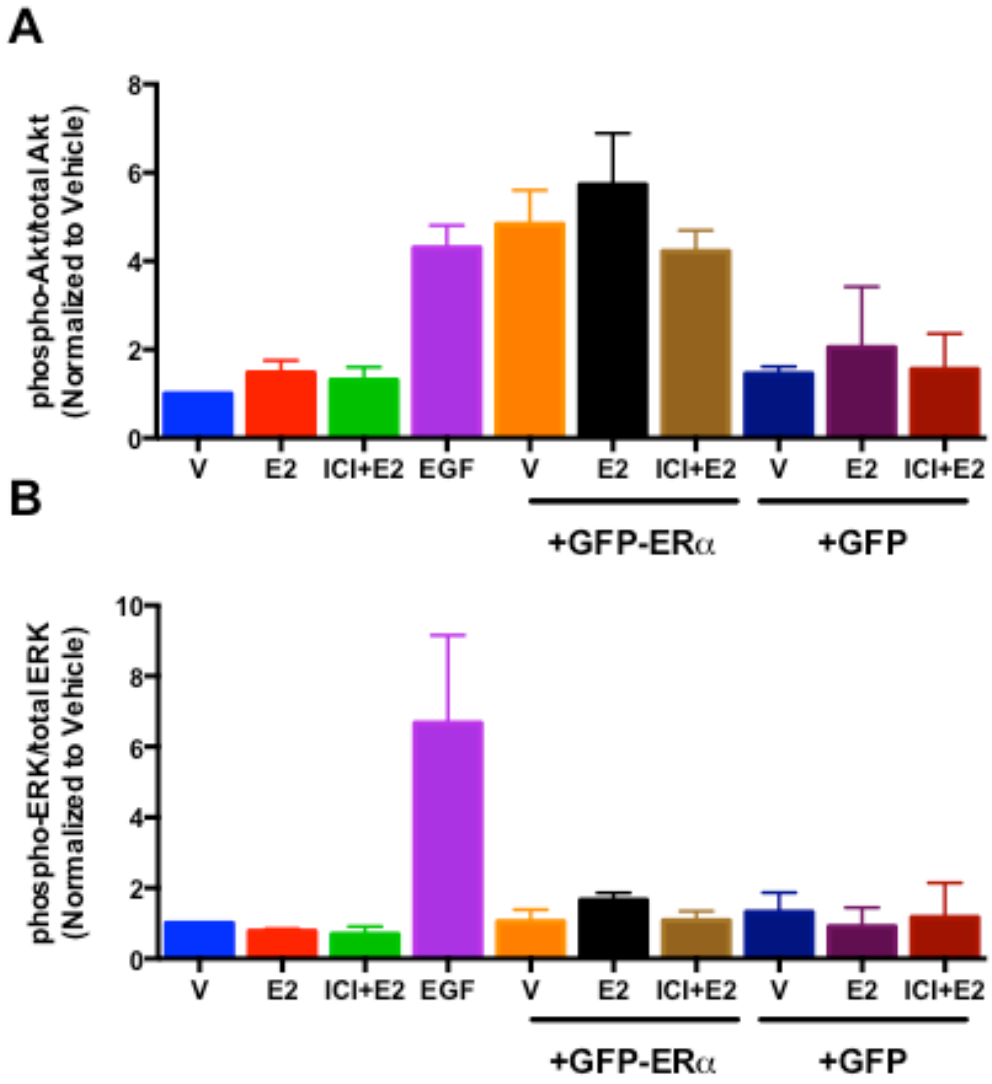

Figure 2.8: EGFP-ERα activation of rapid signaling pathways in NRVMs. Following overexpression with GFP-ERα and treatment with E2 or ER-antagonist, ICI, activation of PI3K or MAPK signaling was assessed. **(A)** Activation of PI3K pathway as measured by pAkt (Thr308) levels relative to total Akt. **(B)**. Activation of MAPK pathway as measured by pERK1/2 (Thr202/Tyr204) levels relative to total ERK1/2.

2.3.5 Alternate ER isoform localization and signaling in cardiac myocytes

Since full-length N-terminally EGFP-tagged ERα was primarily localized to nuclei and capable of activating transcription, but not MAPK signaling, I next assessed whether alternate isoforms of ERα displayed differential localization and/or signaling competencies, as has been observed in other cell types (Kang et al., 2010; Kim et al., 2011; Ohshiro et al., 2012; Wang et al., 2005). To that end, I cloned *EGFP*-tagged *ERα36*, *ERα46*, and *ERα66* into pEGFP-N1 and constructed corresponding adenoviruses so that the localization, transcriptional and rapid signaling competencies of the three different isoforms could be directly compared to one another in cardiac myocytes. For these studies, the EGFP tag was moved from the N-terminus to the C-terminus as previous studies suggest that accessibility of the N-terminus is critical for palmitoylation-regulated targeting of ERα to the cell membrane (Li et al., 2003).

Appropriately sized EGFP C-terminally tagged-ERα36, ERα46, and ERα66 were all expressed robustly in NRVMs (Figure 2.9). Localization of each isoform was assessed following either 1 hour vehicle of 100 pM estrogen treatment using subcellular fractionation as performed for N-terminally tagged EGFP-ERα66 (Figure 2.5). As shown in Figure 2.10, subcellular fractionation of NRVMs revealed similar subcellular localization patterns for all three ERα isoforms. In all cases, regardless of estrogen status, each ERα isoform localized primarily to cardiac myocyte nuclei. This biochemical analysis was also confirmed using high-resolution fluorescence microscopy (Figure 2.11). By co-staining with a nuclear label (DAPI) and a sarcomeric protein marker (F59 antibody, anti-myosin), ERα-EGFP was never observed to co-localize to sarcomeric structures or striated structures like T-tubular membranes, regardless of E2 status.

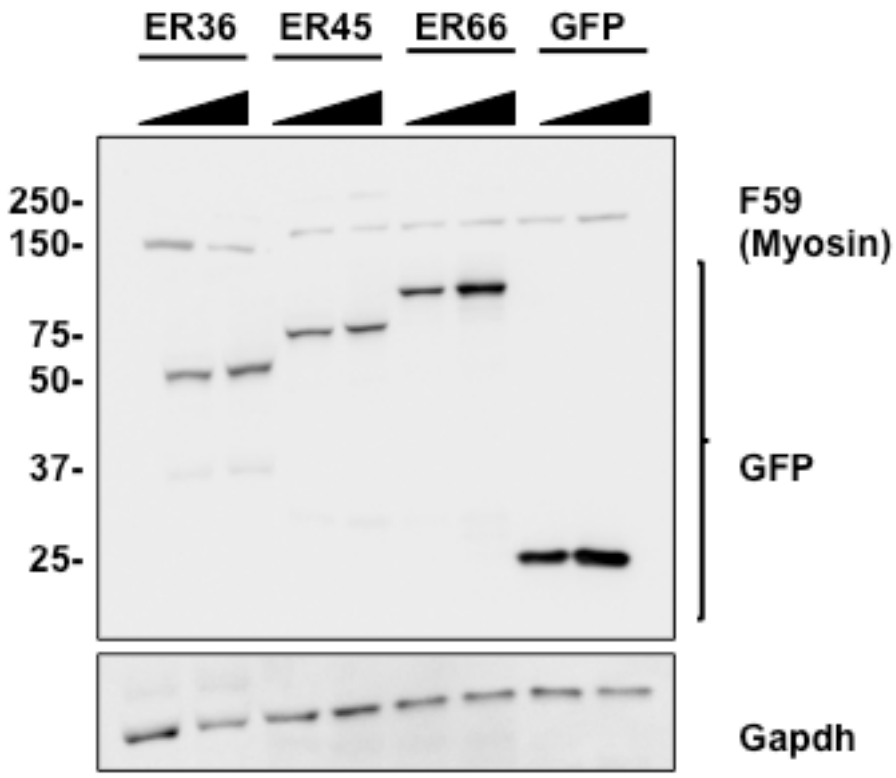

Figure 2.9: ERα36-GFP, ERα46-GFP, and ERα66-GFP overexpression in NRVMs. Immunoblot of whole cell lysates from NRVMs infected with either ERα36-GFP, ERα46-GFP, ERα66-GFP, of GFP-only adenovirus.

Interestingly, sarcomeric proteins co-localized with nuclear proteins during the subcellular fractionation process (Figure 2.12). Co-localization of sarcomeric and nuclear extracts does not allow for resolution of ERα localization to the sarcomere or Z-line using this technique, as had been observed in adult cardiac myocytes using immunofluorescence (Ropero et al., 2006). However, high magnification, high resolution fluorescence microscopy of the EGFP-tagged receptors in NRVMs confirms the primarily nuclear localization pattern (Figure 2.11).

To assess the ability of each ERα spliceform to activate cardiac myocyte-relevant rapid signaling pathways, NRVMs overexpressing each EGFP-tagged ERα isoform were treated briefly (for 5 minutes) with 1 nM estrogen. Cell lysates were then harvested and

probed for activation of either PI3K or MAPK signaling using phosphorylation-specific antibodies. NRVM treatment with EGF was used as a positive control. As shown in Figure 2.13, only treatment with EGF elicited activation of either PI3K or MAPK signaling pathways suggesting that neither ERα isoform is capable of activation of these pathways in neonatal cardiac myocytes. These data also suggest that the activation of pAkt by EGFP-ERα66 observed in Figure 2.8 is either an artifact of increased viral load or specific to N-terminally tagged ERα66.

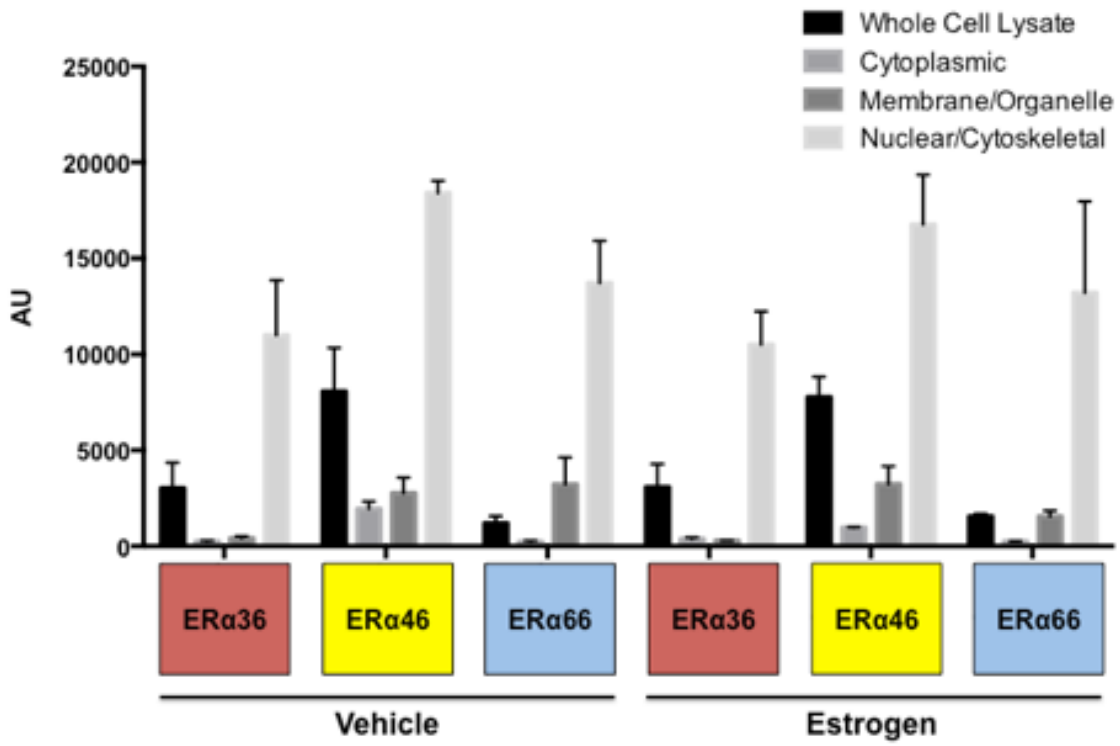

Figure 2.10: Subcellular fractionation of NRVMs and ERα-GFP localization. Following overexpression by adenoviral infection and treatment with either vehicle or 100 pM E2, ERα-EGFP localization was quantified in fractionated cell lysates. Subcellular fraction identity was verified by the presence of either GAPDH (cytosol), Caveolin-3 (Membrane), or Histone-3 (nucleus) (data not shown). Estrogen treatment: 100 pM 1 hour.

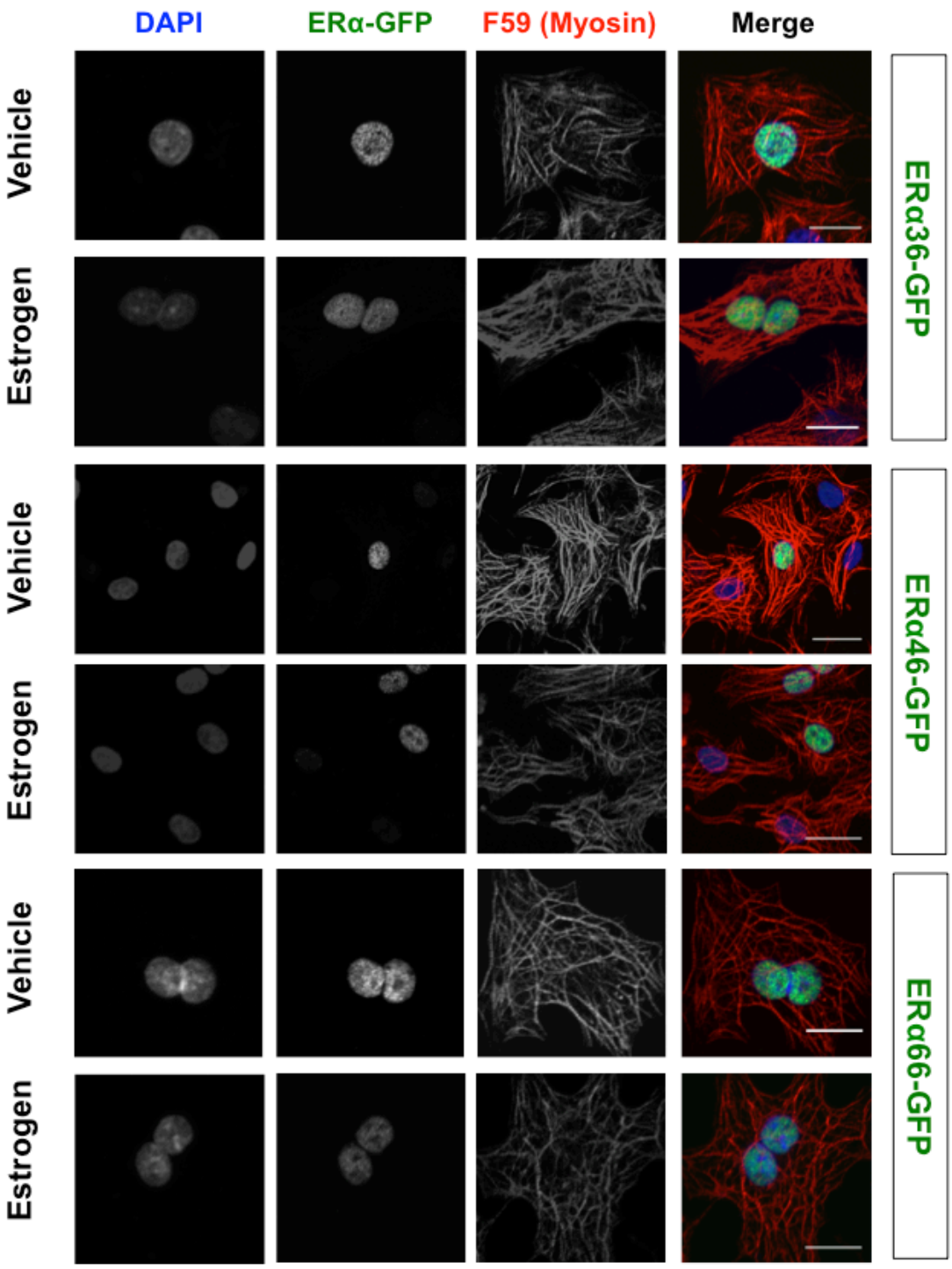

Figure 2.11: ERα-GFP localization in NRVMs. Following overexpression with ERα-GFP isoforms by adenoviral infection and treatment with either vehicle or estrogen, NRVMs were immunostained with F59 (anti-myosin) antibody and Dapi. Localization of ERα-GFP relative to sarcomeres and nuclei was assessed by confocal microscopy. 40x magnification, scale bar: 20 μM.

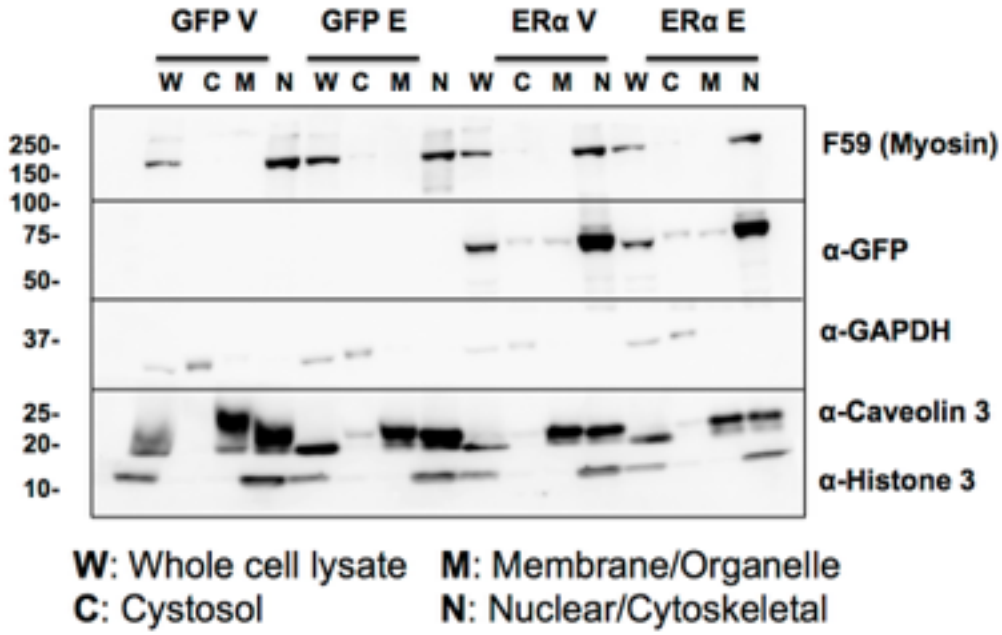

Figure 2.12: Subcellular fractionation of NRVMs, ERα-GFP and sarcomeric protein localization. Following overexpression by adenoviral infection and treatment with either vehicle or 100 pM E2, ERα-EGFP localization was quantified in fractionated cell lysates. Subcellular fraction identity was verified by the presence of either GAPDH (cytosol), Caveolin-3 (Membrane), or Histone-3 (nucleus). F59 antibody was used to determine sarcomeric protein localization relative to other fractions Estrogen treatment: 100 pM 1 hour.

Each ERα isoform was then interrogated for its ability to regulate transcription of a synthetic *ERE* reporter construct. NRVMs were again infected with adenovirus to express each of the three *ERα* isoforms while also concurrently infected with adenovirus containing a synthetic *ERE-luciferase* reporter. Cells were then treated with either 1 nM vehicle or estrogen for 12 hours after which luciferase activity was quantified. As shown in Figure 2.14, luciferase induction varied among *ERα* isoforms with *ERα66-eGFP* demonstrating the greatest induction. As expected based on its truncated N-terminal transactivation domain (Figure 2.1), *ERα46-EGFP* showed comparably lower activation of *ERE-luciferase* similar to what has been observed in other cell types (Figtree et al.,

2003). *ERα36-eGFP* was incapable of inducing *ERE-luciferase,* a finding which is consistent with its lack of both N- and C-terminal transactivation domains and what has been observed in other cell types (Wang et al., 2006).

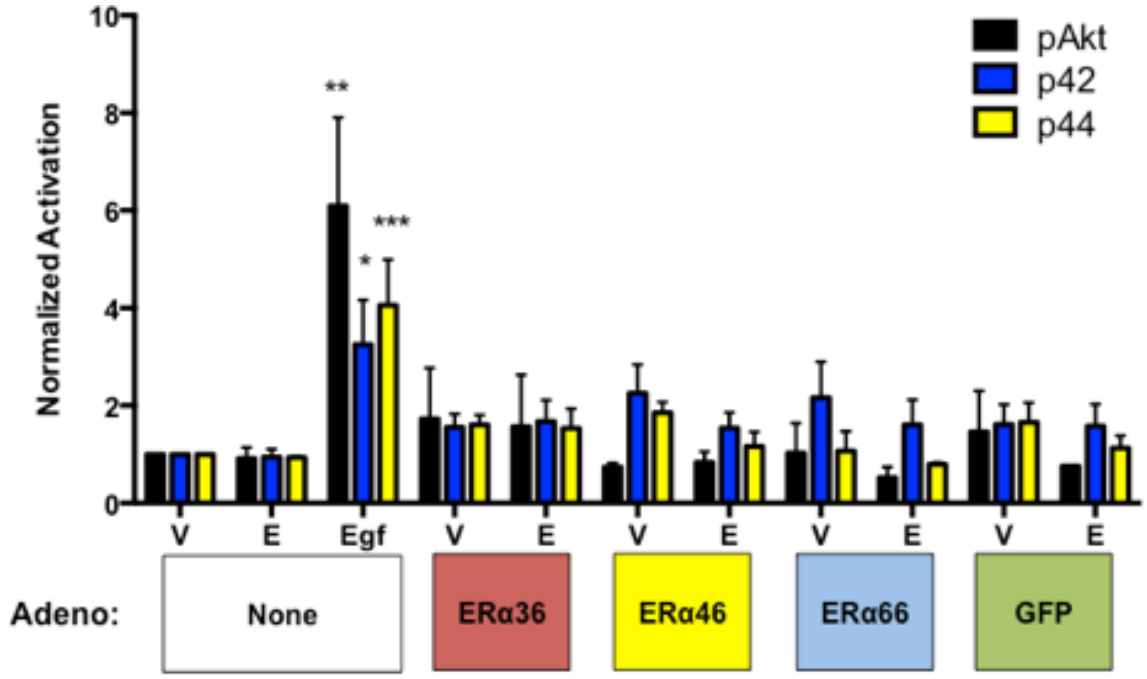

Figure 2.13: Assessment of ERα-EGFP activation of rapid signaling pathways in NRVMs. Following overexpression with ERα-EGFP and 5 minutes treatment with Vehicle (V) or 100 pM E2 (E), activation of PI3K or MAPK signaling was assessed. Activation of PI3K pathway as measured by pAkt (Thr308) levels relative to total Akt. Activation of MAPK pathway as measured by pERK1/2 (Thr202/Tyr204) levels relative to total ERK1/2. Adeno: Adenovirus, EGF: 0.3 μg/mL. **P*<0.05, ***P*<0.01, ****P*<0.001 vs. uninfected, vehicle control.

Discussion

2.4.1 ER expression in cardiac myocytes

To my knowledge, this is the first report of ER expression data in pure populations of isolated neonatal and adult cardiac myocytes using qRT-PCR. Several other groups have reported ER expression and localization patterns using ER-antibodies but antibody specificity remains somewhat unclear (Lizotte et al., 2009; Ropero et al., 2006). These

data suggest an absence of *ERβ* in both neonatal and adult cardiac myocytes despite reported protein expression in ventricular lysates using western blot (Lizotte et al., 2009).

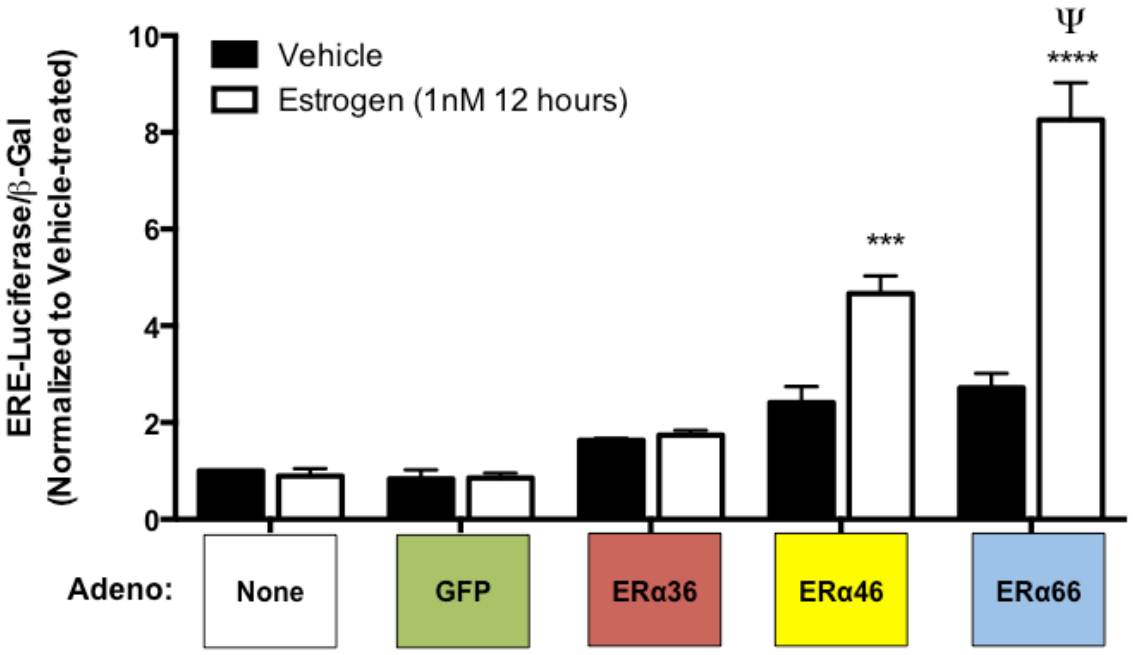

Figure 2.14: Assessment of ERα-EGFP transcriptional activation in NRVMs. Following overexpression with ERα-EGFP and treatment with Vehicle or 1 nM E2, activation of a synthetic *ERE-luciferase* reporter was assessed. ***P<0.001 vs. vehicle treated, . ***P<0.0001 vs. vehicle treated, Ψ P<0.0001 vs. ERα46-EGFP, estrogen treated.

Several models in *ERβ* deficient mice support a role for *ERβ* in the heart and vasculature although our data suggest this role is likely in non-myocyte cells in the heart (Babiker et al., 2006; Pedram et al., 2010). Indeed, many studies support the importance of *ERβ* in non-cardiac myocyte cell and tissue types including cardiac fibroblasts, lung septa, and platelets (Jayachandran et al., 2010; Morani et al., 2006; Pedram et al., 2010). These cell and tissue types can directly and indirectly influence cardiac myocyte function and viability so cardiac phenotypes in mice with systemic loss of *ERβ* may actually be secondary phenotypes (Babiker et al., 2006; Babiker et al., 2007; Pelzer et al., 2005;

Skavdahl et al., 2005).

2.4.2 Full length and alternate ER isoform localization and signaling in cardiac myocytes

Localization of full length and alternately spliced isoforms of *ERα* were carefully assessed in this study. Primary nuclear localization was observed for all three EGFP-tagged *ERα* variants. Although it is conceivable that the EGFP tag could interfere with ERα trafficking, several pieces of evidence support a lack of effect of EGFP on ER localization. First, I observed broad distribution of EGFP alone suggesting EGFP is capable of targeting to all of the subcellular compartments that were assessed. Second, full length *ERα* localization was similar regardless of EGFP tag orientation (amino- or carboxy-terminus). Finally, when a comparably smaller Myc tag was substituted for the N-terminal EGFP tag, nuclear localization was also observed for all three ERα isoforms studied (data not shown).

Others have reported a range of subcellular localizations for *ERα* and its splice variants. Primarily nuclear localization with significant membrane and cytosolic localization of both ERα66 and ERα46 was observed in COS7 fibroblast-like cells and EA.926 immortalized, endothelial cells following overexpression with GFP-tagged constructs (Figtree et al., 2003). Another group reported enrichment of ERα46 in the cytosol and plasma membrane relative to the nucleus in EA.926 cells (Li et al., 2003). Our results in cardiac myocytes are inconsistent with these findings as the majority of ERα46 and ERα66 was localized in the nucleus.

The most recently discovered *ERα* variant, *ERα36* appears to be functionally incompetent in cardiac myocytes, at least in its ability to regulate transcription and induction of PI3K or MAPK signaling. This does not rule out another mechanism of

ERα36 action in NRVMs or adult cardiac myocytes or a human-specific cardiac myocyte function for this variant. Importantly, *ERα36* transcript expression has been solely identified in human tissues (Wang et al., 2005; Zheng et al., 2010). A corresponding mouse or rat transcript has yet to be cloned. The nuclear localization pattern of ERα36 (Figures 2.10 and 2.11) is consistent with its retention of the DNA binding domain and nuclear localization sequence while its inability to activate transcription (Figure 2.14) agrees with its lack of N- and C-terminal transactivation domains. Nevertheless, my findings using an EGFP-tagged *ERα36* construct do not recapitulate membrane and cytoplasmic localization patterns observed by others using immunofluorescence or subcellular fractionation in conjunction with isoform-targeted ERα antibodies (Lee et al., 2008; Wang et al., 2006).

There is ample precedent for the importance of nongenomic ERα signaling in the heart. Recent generation of a transgenic mouse in which membrane-associated ERα signaling is disrupted revealed the importance of membrane localized ERα in protecting the heart from vascular injury (Bernelot Moens et al., 2012; Lu et al., 2004). Cells isolated from transgenic mice that are unable to initiate membrane ERα signaling were deficient in their ability to activate E2-dependent pAkt and pERK activation suggesting the importance of these two pathways in mediating the effect of E2-ERα rapid-signaling induced cardioprotection. Data presented in this chapter point to the importance of non-myocyte cardiac cell types in facilitating this effect.

The inability of each ERα variant to regulate rapid E2 signaling effects does however agree with the lack of extra-nuclear ERα in cardiac myocytes compared to what has been previously reported for other cell types. These results together support a

primarily nuclear function for ERα in cardiac myocytes. The relevant gene targets for ERα46 and ERα66 in cardiac myocytes warrant further investigation and may reveal novel cardiac myocyte-specific targets for E2-ERα.

Although EGFP-tagged *ERα* isoforms could be robustly expressed in NRVMs, their relevance to adult cardiac myocyte biology remains in question. NRVMs were chosen for cardiac myocyte studies due to the extremely low endogenous levels of *ERα* (Figure 2.2) but functional and biological similarity to adult cardiac myocytes where expression of *ERα* is much higher (Figure 2.2). However, ERα46 and ERα36 protein expression have been observed by others using western blot of lysates from adult cardiac myocytes or total ventricular extracts (Irsik et al., 2013; Ropero et al., 2006). In my hands, the antibodies used in these studies were not particularly specific so it is unclear how much of each isoform exists in adult cardiac myocytes. From my studies, which follow fluorescently tagged ER's in both live and fixed cells, full length ERα is the functionally relevant isoform for cardiac myocytes and its principal mechanism of signaling is through transcriptional activation.

Chapter 3

The *in vivo* requirement for Estrogen-Receptor-α in cardiac myocytes

Abstract

Despite decades of clinical and biological investigation into the *in vivo* role of estrogen signaling in the heart, the cardiac-myocyte requirement of estrogen signaling is unknown. To address this cell type-specific requirement, a cardiac myocyte-specific knockout of the primary cardiac myocyte ER, *ERα* was generated using Cre-loxP gene-targeting technology. The molecular and functional consequences of *ERα* deletion were assessed *in vivo* in males and female at baseline and following either physiological or pathological pro-hypertrophic stimulation. *ERα* conditional knockout mice were histologically and functionally similar to their control, *ERα* floxed littermates at 3 months of age at baseline but functionally declined with age, similar to heterozygous *ERα* conditional knockout mice. Molecularly, *ERα* conditional knockout female hearts displayed transcriptional evidence of cardiac remodeling. Physiological cardiac hypertrophy was impaired in female *ERα* conditional knockout mice though function was not changed. Pathological cardiac stress induced adverse remodeling in female *ERα* conditional knockout mice. The aging phenotype observed in *ERα* cardiac myocyte knockout mice can be recapitulated in mice expressing cardiac myocyte-specific *Cre* recombinase in the absence of a floxed *ERα* allele suggesting *ERα* deletion in cardiac myocytes does not have profound effects on baseline cardiac function in young mice.

3.1 Introduction

While both male and female hearts undergo remodeling, sexual dimorphisms exist

with respect to the hypertrophic cardiac response (Konhilas et al., 2004). Historically, the decreased risk of cardiovascular disease in pre-menopausal females led to the investigation of sex hormones as mediators of cardiac function (McKee et al., 1971; Mendelsohn and Karas, 2005). Indeed, both genetic and hormone replacement studies have demonstrated that the sex hormone, 17β-estradiol (estrogen or E2), plays a key role in cardiac hypertrophy (Babiker et al., 2006; Booth et al., 2005; Patten and Karas, 2006). However, the nature of this role is confounded by E2's involvement in diverse physiological processes including maintenance of reproductive function and vascular biology. Further support for a specific role for E2-ERα in cardiac remodeling is evidenced by the dynamic regulation of *ERα* transcript in murine models of cardiac adaptation (Figure 3.1).

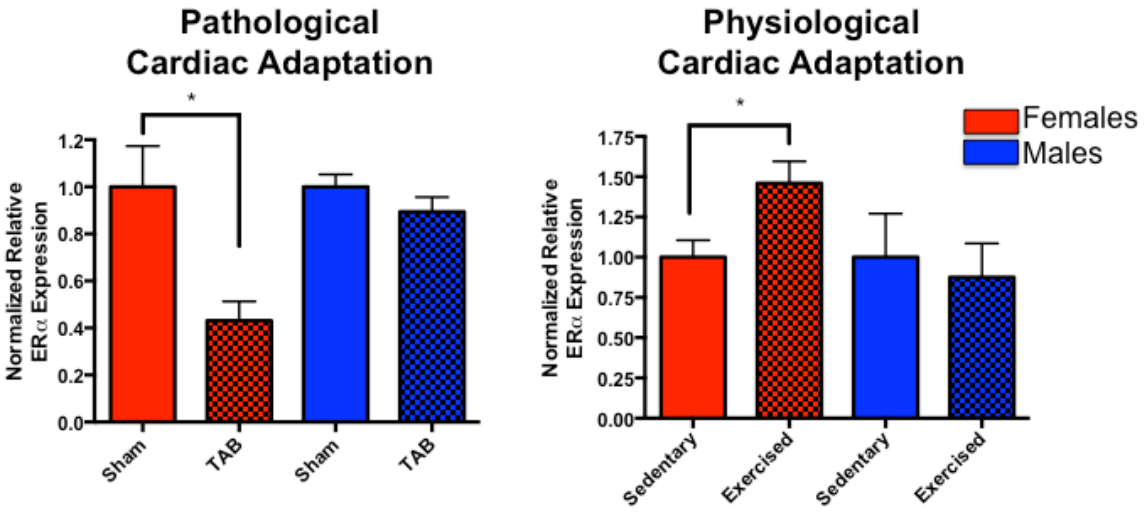

Figure 3.1: Left ventricular *ERα* expression during cardiac adaptation. (A) Normalized, relative *ERα* expression in sham-operated or TAB mice. **(B)** Normalized, relative *ERα* expression in sedentary or voluntarily exercised mice (wheel running 3 weeks). N=4-5/group, **P*<0.05, vs. sex-matched, age-matched control. TAB: Trans-aortic banding.

As shown, in females left ventricular *ERα* expression is downregulated in a common model of pathological remodeling, trans-aortic banding (TAB) (Figure 3.1A). In contrast, left ventricular *ERα* expression is upregulated in females in a common model of physiological cardiac remodeling, exercise (Figure 3.1B). Importantly, E2-ERα is not sufficient to induce cardiac hypertrophy in isolated neonatal cardiac myocytes (Figure 3.2), however the requirement for E2-ERα signaling in cardiac myocytes is unknown.

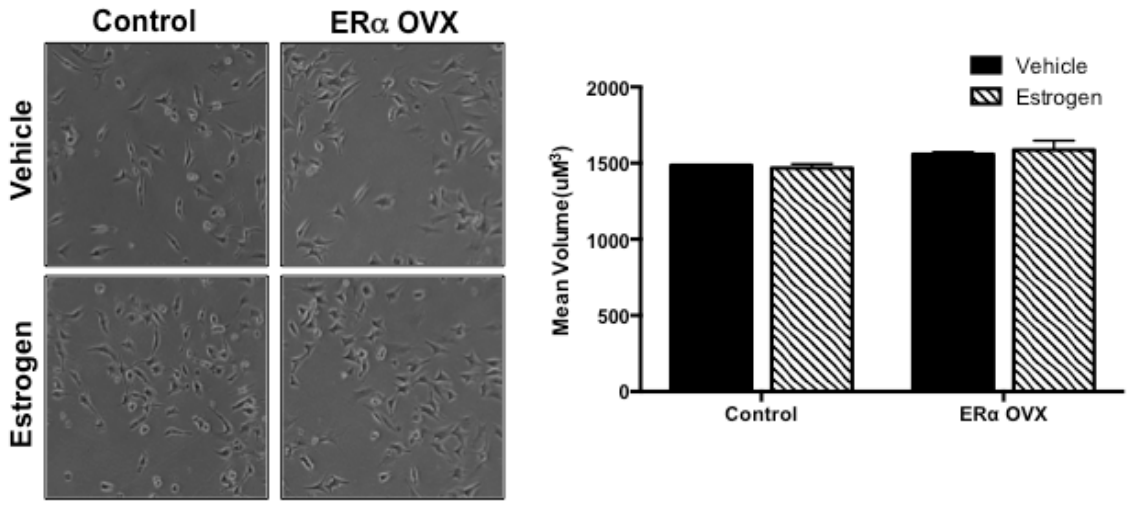

Figure 3.2: E2-ERα is not sufficient to induce cardiac hypertrophy in isolated neonatal cardiac myocytes. Neonatal rat ventricular myocytes (NRVMs) were isolated and infected with adenovirus containing either *EGFP* or *EGFP-ERα*. Following expression of *EGFP* or *EGFP-ERα* cells were treated with vehicle or E2 for 24 hours and cell size was measured. No differences in cell size were observed between control (EGFP) and E2-ERα conditions. OVX: overexpression.

The sexually dimorphic response to exercise supports this model for the investigation of the involvement of E2-ERα signaling in physiological hypertrophic remodeling. For example, females achieve a greater increase in cardiac mass than do males when heart size is normalized to the total amount of distance run (Konhilas et al., 2004). While pathological remodeling of the myocardium is also sexually dimorphic, several distinguishing hallmarks that result from differential signaling pathway activation

distinguish pathologic from physiological remodeling. During pathological remodeling, a program of fetal gene expression is induced, which changes myosin composition and leads to impaired myocyte ATPase activity (Mahdavi et al., 1984). Extracellular matrix (ECM) proteins accumulate in the extracellular space causing mechanical stiffness in the myocardium (Brower et al., 2006). However, exercise-induced cardiac remodeling results in hypertrophy and functional maintenance or improvement without the same alterations in gene expression or accumulation of fibrotic matrix (Jin et al., 2000). Whether either ER plays an exclusive or dual role in these hypertrophic processes is as yet unknown at the cardiac myocyte level. Based on results from Chapter 3, ERβ is unlikely to play a role in this process given its lack of abundance, or extremely low abundance in cardiac myocytes.

Chronic treatment with the β-adrenergic agonist, isoproterenol (Iso), leads to typical, pathological remodeling through several different mechanisms including activation of angiotensin II, which stimulates cardiac fibroblasts to produce ECM proteins (Crawford et al., 1994; Grimm et al., 1998). Both *in vivo* and *in vitro* models of Iso treatment are routinely used to model pathological hypertrophy (reviewed in (Osadchii, 2007)) and are employed in this chapter to assess the requirement of E2-ERα signaling for this process. Interestingly, recent evidence from the Leinwand lab demonstrates that in the absence of all endogenous estrogens, due to aromatase KO, the hypertrophic response to Iso is modified in females such that it resembles the wild-type (WT) male response (Haines et al., 2012a).

While total KO models of both ERs have been studied extensively, the diverse systemic roles of E2-ER signaling confound a clear understanding of the role for E2-ER

signaling in cardiac hypertrophy in these mice. Not only are total ERKO mice infertile (Lubahn et al., 1993) but they also have increased circulating E2 (Couse et al., 1995). Abnormal primary and secondary cardiovascular effects make difficult the interpretation of the cardiac phenotype in the total KO mice (Lindner et al., 1998; Venkov et al., 1996; Zhu et al., 2002). These observations together highlight the need for a cardiac myocyte-specific *ERα* knockout model to determine the explicit role for E2-ERα signaling in the myocardium. In this chapter, the baseline cardiac phenotype and response of the ERα cKO heart to exercise, a physiological stimulus, and isoproterenol, a pathological stimulus are characterized. These data reveal the specific roles for ERα-mediated E2 signaling in baseline cardiac biology and cardiac adaptation and add to the growing body of understanding regarding sexually dimorphic clinical outcomes with respect to CVD. Ultimately, a more refined comprehension of E2-ER signaling can inform improved use of hormone therapies for post-menopausal women.

3.2 Materials and methods

Hypertrophy in Neonatal Rat Ventricular Myocytes (NRVMs)

Neonatal rat ventricular myocytes (NRVMs) were isolated from 1 day old pups as previously described (Maass and Buvoli, 2007). Cells were infected with either control (EGFP only) or EGFP-*ERα* containing adenovirus for 24 hours followed by either treatment with vehicle or 1 nM estrogen. Overexpression was confirmed by GFP fluorescence. Following 24 hours treatment, cell volume was measured in triplicate using a Multisizer 3™ Coulter Counter® (Beckman Coulter), as previously described (Cosper et al., 2012).

Transgenic Mice

All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Colorado at Boulder. Mice were fed *ad libitum* standard rodent chow and housed in a facility with a 12 hour light, 12 hour dark cycle. α*MyHC-Cre* mice were backcrossed into the C57Bl/6J background for at least 10 generations and bred using either heterozygote ($ERα^{lox/+}$; $αMyHC:Cre^{+/-}$) or homozygous *ERα* cKO ($ERα^{lox/lox}$; $αMyHC:Cre^{+/-}$) males and *ERα floxed* ($ERα^{lox/lox}$; $αMyHC:Cre^{-/-}$) females. Genotyping was performed using *ERα* and *αMyHC:Cre* primers (Appendix I) and re-confirmed post-mortem.

Transthoracic echocardiography

Non-invasive echocardiographic images and measurements were made using the Philips Sonos 5500 system. Mice were placed on a heating pad and maintained on 2% isoflurane via spontaneous inhalation. The mouse's fur was first removed from the ribcage using a depilatory cream, and an image-potentiating gel was then applied for image acquisition. M-(motion) mode images were captured for each animal at the level of the papillary muscles (A2 view). Left ventricular dimensions and function were calculated from the M-mode images, where wall thickness of the anterior and posterior walls and the chamber diameter were measured using the ASE leading edge convention.

Voluntary wheel running

3-month-old mice were subjected to voluntary wheel running for 21 days as previously described (Konhilas et al., 2004). Briefly, wheels were 11.5 cm in diameter with a 5.0 cm wide running surface (model 6208; PetsMart) equipped with a digital magnetic counter (model BC 600; Sigma Sport) activated upon wheel rotation. For each litter, mice were randomly assigned to the particular exercise duration or sedentary

littermate control. All animals were given water and standard pelleted rodent feed *ad libitum*. Daily exercise values for time and distance run were recorded for each exercised animal throughout the duration of the exercise period. Mice underwent echocardiography one day prior to commencing the exercise program and on the final day of the exercise program. Following the 21-day period, mice were euthanized by cervical dislocation under inhaled anesthesia. Hearts were excised, weighed, and washed in ice-cold PBS. The left ventricle was then isolated and partitioned into 3 pieces for RNA/protein/DNA extraction and flash frozen in liquid Nitrogen for subsequent analyses.

Isoproterenol treatment

3-month-old mice were treated for one week with isoproterenol (Iso) as described (Haines et al., 2012a). Briefly, Iso was chronically administered to mice using a surgically implanted subcutaneous miniosmotic pump (Alzet 2001) that released Iso in 0.9% NaCl, at a rate of 30 mg/kg of body weight per day. Control pumps delivered 0.9% NaCl solution alone. Seven days after implantation of pumps, mice underwent echocardiography. The following day mice were euthanized by cervical dislocation under inhaled anesthesia. Hearts were excised, weighed, and washed in ice-cold PBS. The left ventricle was then isolated and partitioned into 3 pieces for RNA/protein/DNA extraction and flash frozen in liquid Nitrogen for subsequent analyses.

Gene expression

Total RNA was purified from left ventricles using TRI Reagent (Ambion) according to the manufacturer's protocol. cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen) and random hexamer primers. Gene expression was determined by qRT-PCR using SYBR Green dye, gene specific primer sets (Appendix I)

and a Bio-Rad CFX-96 Real-Time PCR system.

Western blot

Left ventricles were homogenized in 50mM Tris pH 8.0, 150mM NaCl, 1% NP40, 0.5% Na-deoxycholate, 0.1% SDS, complete protease inhibitor cocktail (Roche) and the following phosphatase inhibitors: 1mM PMSF, 2mM NaF, 2mM NaPPi, 1mM Beta-Glycerophosphate, 1mM Na-molybdate dihydrate, and 1mM Na-Orthovanadate. 10 µg of protein was resolved on a 10% SDS-PAGE gel and probed with the following antibodies: GAPDH (Cell Signaling Technologies 2118s), phospho-ERK (Cell Signaling Technologies 9101s), total ERK (Cell Signaling Technologies 9102s), pAkt (Cell Signaling 9275s), Akt (Cell Signaling 9272).

Histology

Hearts were fixed in 10% buffered formalin overnight and then transferred to 70% ethanol. Fixed hearts were rehydrated, embedded in paraffin, sectioned longitudinally, and stained with hematoxylin and eosin.

Data and statistical analysis

Data are presented as mean ± SEM. Differences between groups were evaluated for statistical significance using Student's two-tailed t test (two groups) or one-way ANOVA (more than two groups) followed by Tukey's post-hoc test for pairwise comparisons. For comparisons between multiple treatments and groups, two-way ANOVA was performed followed by Tukey's post-hoc test. *P* values less than 0.05 were considered significant unless otherwise noted.

3.3 Results

3.3.1 Conditional deletion of ERα *in cardiac myocytes*

In order to determine the role of *ERα* in cardiac myocytes, I conditionally deleted *ERα* from these cells using Cre/loxP technology. Conditional deletion in cardiac myocytes was achieved by crossing floxed *ERα* mice ($ERα^{lox/lox}$) with mice that express *Cre* recombinase under the control of a cardiac myocyte-specific promoter. Briefly, floxed *ERα* transgenic mice were obtained in which the endogenous *ERα* locus is replaced with an allele where *loxP* sites have been engineered into the flanking region of exon 3 such that upon recombination, exon 3 is deleted and a premature stop codon is introduced when exons 2 and 4 are spliced together (Dupont et al., 2000). Mice expressing *Cre* recombinase under the cardiac myocyte-specific *alpha myosin heavy-chain* (*αMyHC*) promoter were previously generated and demonstrated to express abundant *Cre* recombinase solely in cardiac myocytes (Agah et al., 1997).

In order to generate control ($ERα^{lox/lox}$; $αMyHC:Cre^{-/-}$) and *ERα* conditional knockout mice (*ERα* cKO) ($ERα^{lox/lox}$; $αMyHC:Cre^{+/-}$) as littermate controls, female $ERα^{lox/lox}$ mice were bred with male *ERα* cKO mice. Based on Mendelian inheritance, 50% of offspring should be wild-type (WT) controls and 50% should *ERα* cKO. Observed genotypes of offspring did not differ from expected genotypes with 43% of male and 51% of female offspring being WT and the remainder *ERα* cKO (n=73 mice).

ERα depletion was confirmed by qRT-PCR of RNA isolated from total left ventricular homogenates (Figure 3.3). Approximately 50% of left ventricular *ERα* transcript was reduced in both heterozygous ($ERα^{lox/+}$; $αMyHC:Cre^{+/-}$) and homozygous ($ERα^{lox/lox}$; $αMyHC:Cre^{+/-}$) *ERα* cKO mice. Given expression of *ERα* in other cell types in myocardium including fibroblasts and endothelial cells (Figtree et al., 2003; Pare et al.,

2002; Venkov et al., 1996), it was not surprising that *ERα* was not 100% reduced in *ERα* cKO mice.

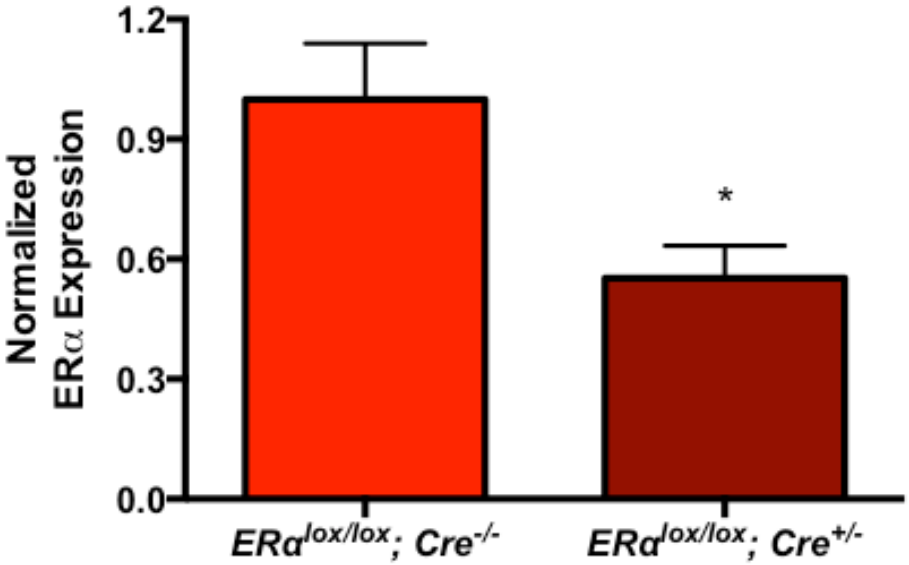

Figure 3.3: Left ventricular *ERα* mRNA expression. Left ventricular *ERα* mRNA expression is reduced by ~50% in female *ERα* cKO mice. N=3-5/group, **P*<0.05.

3.3.2 Baseline cardiac phenotype in ERα *cKO mice*

To determine the functional consequences of *ERα* deletion in cardiac myocytes, serial echocardiography was performed on male and female WT ($ERα^{lox/lox}$) and *ERα* cKO ($ERα^{lox/lox}$*; αMyHC:*$Cre^{+/-}$) mice at 3, 6, and 9 months of age. Although *ERα* cKO mice were functionally indistinguishable from WT siblings at 3 months of age, by 6 months of age cardiac function was modestly, but significantly decreased in both male and female *ERα* cKO mice (Figure 3.4). A progressive decline in cardiac function was also observed in 9-month-old *ERα* cKO mice (Figure 3.4). Decreasing cardiac function was accompanied by progressive, systolic cardiac dilation in both male and female *ERα* cKO mice (Figure 3.5). Although differences in survival were not observed in mice younger than a year, survival in both heterozygous, *ERα* cKO mice ($ERα^{lox/+}$*;*

αMyHC:Cre$^{+/-}$) and homozygous *ERα* cKO mice (*ERα*$^{lox/lox}$; *αMyHC:Cre*$^{+/-}$) significantly differed from control mice (*ERα*$^{lox/lox}$; *αMyHC:Cre*$^{-/-}$) (Figure 3.6).

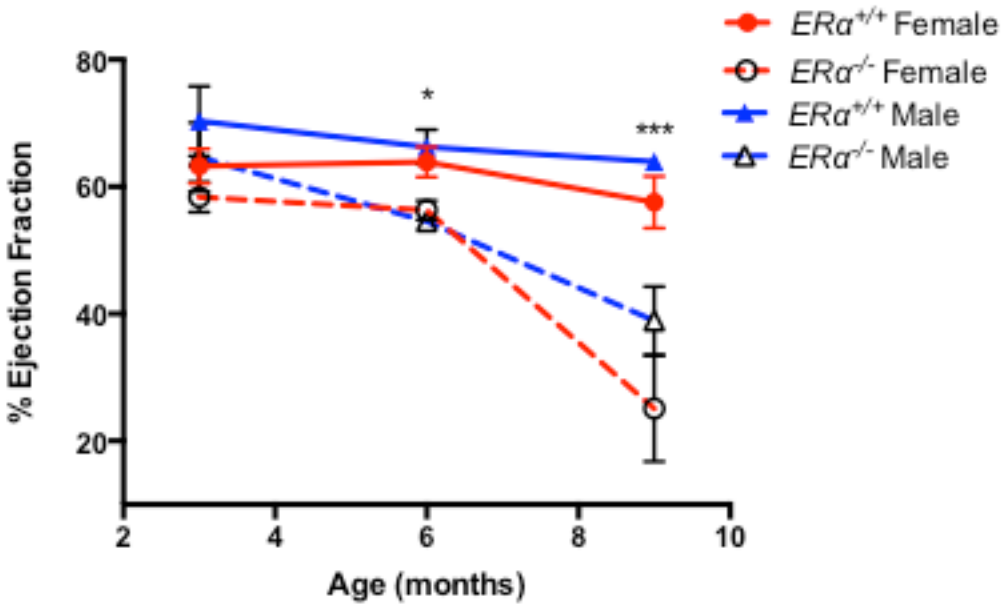

Figure 3.4: Cardiac function over time in *ERα* WT and cKO mice. Left ventricular % Ejection Fraction in 3, 6, and 9 months old male and female control (*ERα*$^{lox/lox}$; *αMyHC:Cre*$^{+/-}$) and cKO (*ERα*$^{lox/lox}$; *αMyHC:Cre*$^{+/-}$) mice. N=3-4/group, **P*<0.05, ****P*<0.001 vs. sex-matched, age-matched controls.

Since male and female mice of all genotypes were functionally indistinguishable at 3 months, I performed a histological analysis of female hearts at this timepoint to determine whether subtle differences exist at the structural or cellular level. As shown in Figure 3.7 and in agreement with the lack of functional discrepancies between WT and *ERα* cKO mice, no gross differences were observed between control and *ERα* cKO mice using hematoxylin and eosin staining.

To determine the molecular consequences of *ERα* deletion in cardiac myocytes, I analyzed left ventricular gene expression and signaling pathway activation in young (3 month old) *ERα* cKO mice, prior to the functional cardiac decline observed at 6 months

(Figure 3.4). mRNA expression was assessed for a panel of genes whose expression is known to be regulated in pathological settings prior to functional changes (Villarreal and Dillmann, 1992). In female mice, several of the genes measured were increased in *ERα* cKO ventricles compared to ventricles from age and sex-matched control mice, indicating activation of pathological signaling pathways (Figure 3.8A). Anti-hypertrophic *Atrial naturietic peptide*, *Anp*, was notably increased in *ERα* cKO ventricular myocardium and cardiac myosin expression was shifted toward a pathological ratio (increased βMyHC) (Figure 3.8A). Modest increases in *Collagen* expression were also observed in *ERα* cKO females (Figure 3.8A). Interestingly, pathological gene expression markers were not correspondingly upregulated in *ERα* cKO males (Figure 3.8B). Modest upregulation of *Anp* was the sole indication of cardiac pathology in *ERα* cKO males indicating a sexually dimorphic effect of cardiac myocyte deletion of *ERα* (Figure 3.8B).

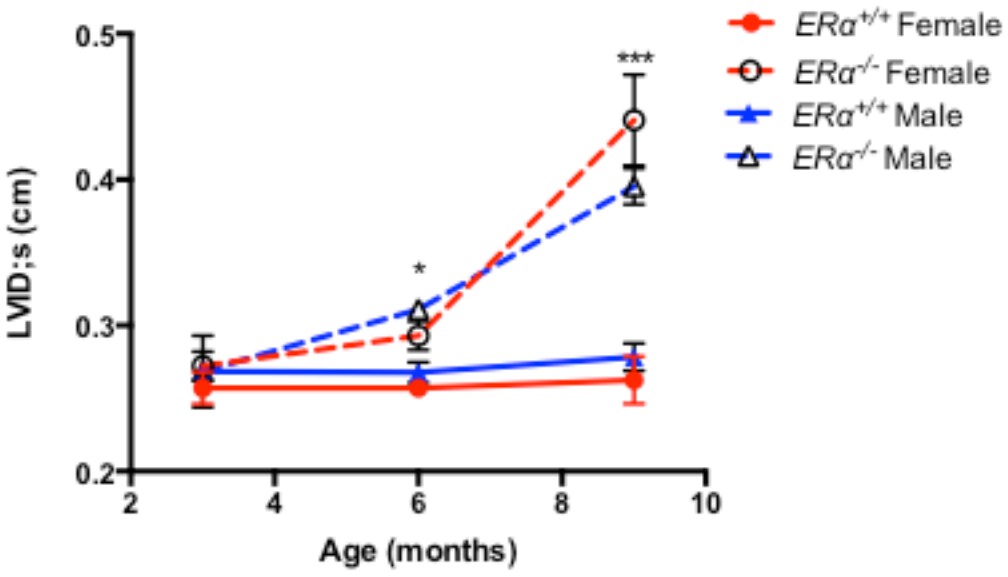

Figure 3.5: Left ventricular internal diameter;systole (LVID;s) over time in *ERα* WT and cKO mice. LVID;s in 3, 6, and 9 months old male and female control (*ERα*$^{lox/lox}$; *αMyHC:Cre*$^{-/-}$) and cKO (*ERα*$^{lox/lox}$; *αMyHC:Cre*$^{+/-}$) mice. N=3-4/group, **P*<0.05, ****P*<0.001 vs. sex-matched, age matched-control.

Given the increased severity of the molecular phenotype observed in female *ERα*

cKO mice compared to male, I next asked whether left ventricular myocardial signaling pathway activation was also affected in female *ERα* cKO mice compared to wild-type control mice. Interestingly, despite known interaction of E2-ERα with two important myocardial signaling pathways (PI3K/Akt and pERK/MAPK) (Nuedling et al., 1999b; Simoncini et al., 2000), no changes in activation of these pathways were observed in *ERα* cKO hearts compared to those of control mice (Figure 3.9) suggesting either lack of an effect of E2-ERα on these pathways in cardiac myocytes or compensatory mechanisms of activation. Together these data support a minor role for *ERα* in cardiac myocytes in maintaining the overall physiologic health of the myocyte.

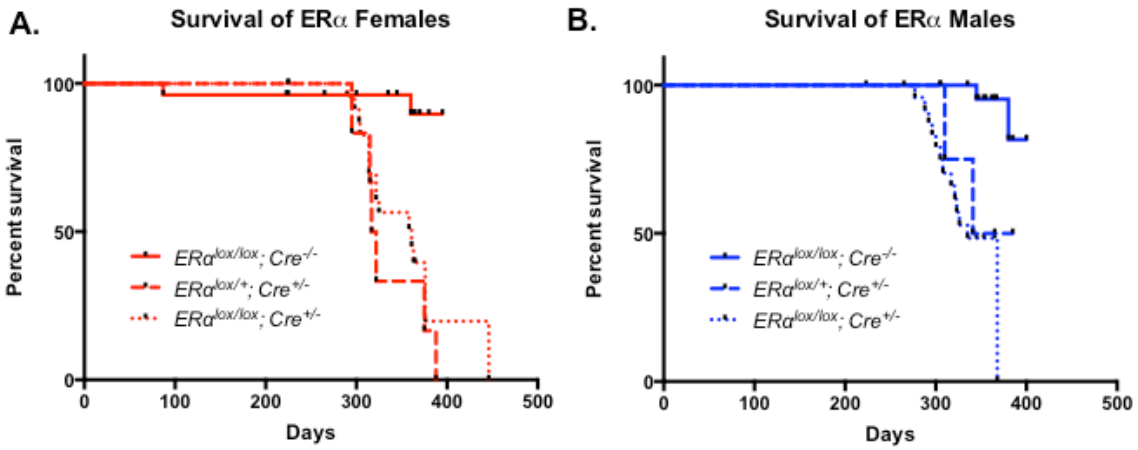

Figure 3.6: Longitudinal survival of WT and *ERα* cKO mice. Kaplan-Meier survival curves for female **(A)** and male **(B)** WT ($ERα^{lox/lox}$; $αMyHC:Cre^{-/-}$) and heterozygous ($ERα^{lox/+}$; $αMyHC:Cre^{+/-}$), and cKO ($ERα^{lox/lox}$; $αMyHC:Cre^{+/-}$) mice. Log-rank (Mantel-Cox) test: N=20-25/group except for $ERα^{lox/+}$; $αMyHC:Cre^{+/-}$ N=7/group, $P < 0.01$ for both heterozygous and cKO vs. WT for both females and males.

3.3.3 Response of ERα *cKO mice to physiological cardiac stress*

Although 3-month-old female *ERα* cKO only displayed modest aberrations in cardiac gene expression, I hypothesized that *ERα* could be required for myocyte adaptation to either physiological or pathological stressors, which rely heavily on

activation of PI3K and MAPK signaling pathway activation (Clerk and Sugden, 2006; McMullen et al., 2003). To test this hypothesis, I subjected female mice to 21 days of voluntary wheel running. Prior to the start of the study and following the 21-day period, cardiac function and morphology was assessed using echocardiography. As shown in Figure 3.10A, although their age-matched, WT counterparts achieved significant cardiac hypertrophy following the 21-day period, female *ERα* cKO mice did not achieve significant cardiac hypertrophy. However, cardiac function did not differ significantly between genotypes (Figure 3.10B). Thus, *ERα* is required in cardiac myocytes for physiological cardiac hypertrophy in a voluntary exercise model.

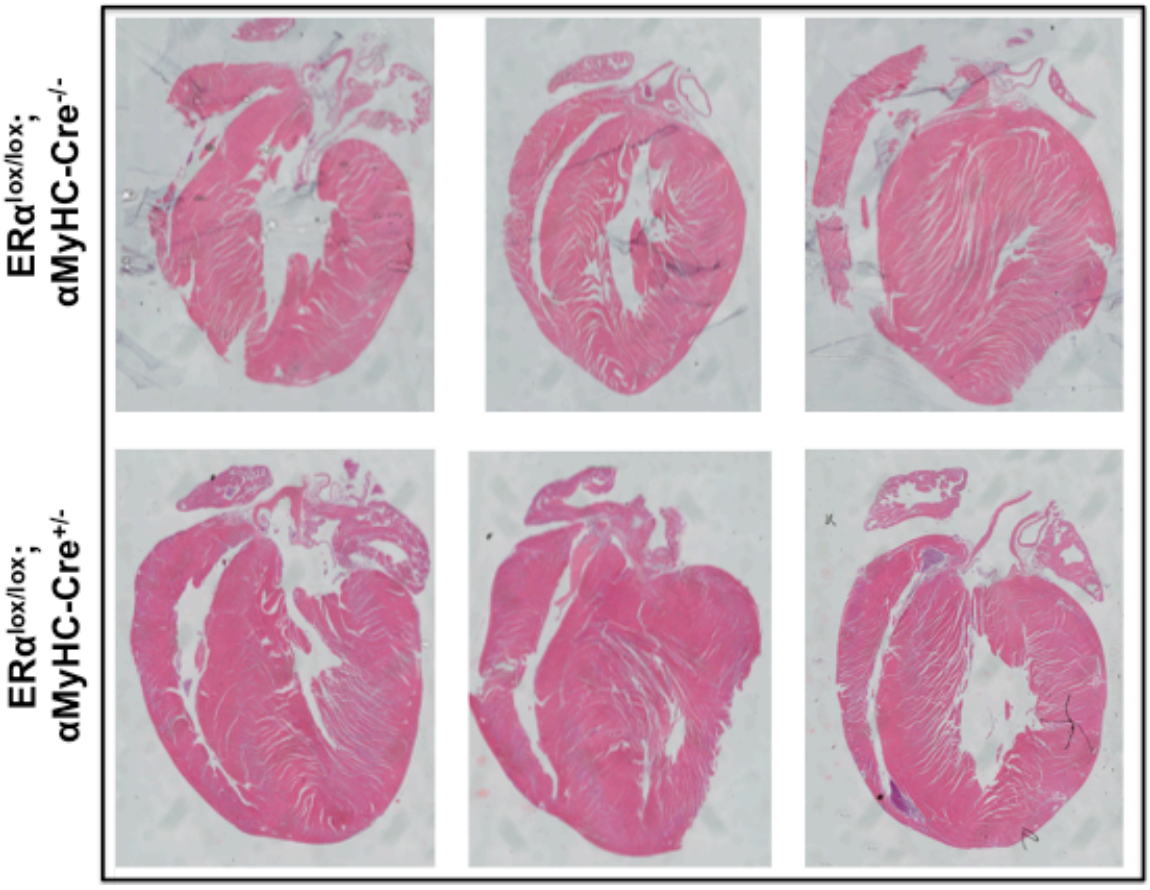

Figure 3.7: Histological analysis of WT and *ERα* cKO hearts. No gross histological differences were observed between female WT (*ERα*$^{lox/lox}$;

αMyHC:Cre$^{-/-}$) and and cKO (*ERα*$^{lox/lox}$*; αMyHC:Cre*$^{+/-}$) hearts. Hematoxylin and eosin staining.

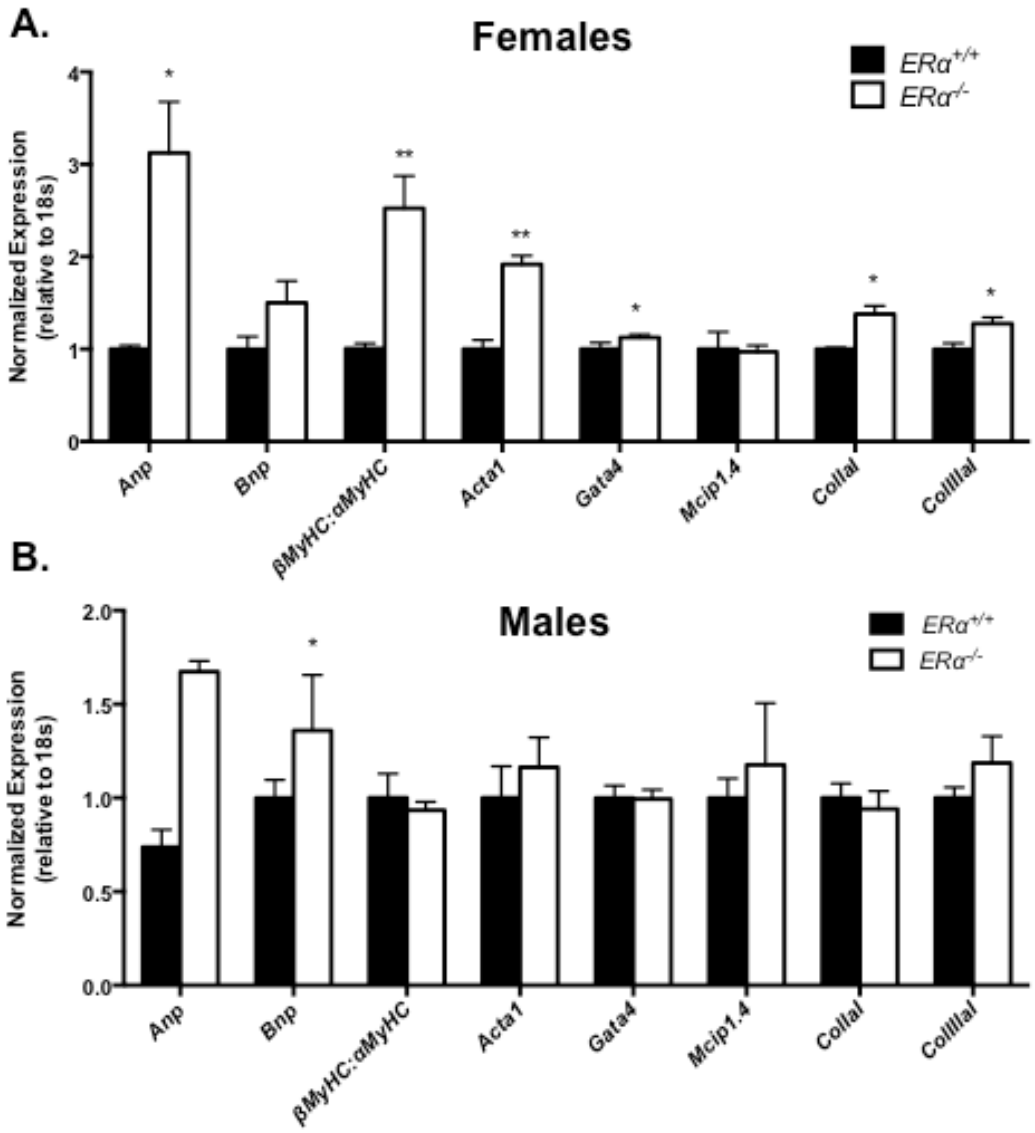

Figure 3.8: Myocardial ventricular gene expression in 3-month-old *ERα* mice. Left ventricular mRNA expression for myocardial markers of remodeling in female **(A)** and male **(B)** WT (*ERα*$^{lox/lox}$*; αMyHC:Cre*$^{-/-}$) and and cKO (*ERα*$^{lox/lox}$*; αMyHC:Cre*$^{+/-}$) mice. N=4-5/group, **P*<0.05, ***P*<0.01 vs. sex-matched control.

3.3.4 Response of ERα *cKO mice to pathological cardiac stress*

Finally, to assess whether *ERα* is required for cardiac myocyte response to a pathological stressor, female WT, heterozygous *ERα* cKO, and *ERα* cKO mice were

treated with either vehicle or 30mg/kg/day isoproterenol (Iso) via subcutaneous osmotic minipump. Mice of all three genotypes achieved comparable hypertrophy following 7 days treatment (data not shown) however cardiac function and morphology were adversely affected in mice lacking one copy of *ERα* (Figure 3.11). Systolic cardiac dilation and depressed % Fractional Shortening were apparent in *ERα* heterozygous cKO mice ($ERα^{lox/+}; Cre^{+/-}$). Although cardiac function in *ERα* cKO mice was not statistically different from that of WT mice, function also did not differ between *ERα* heterozygous and cKO mice, suggesting increased variability in the *ERα* cKO group. These data further support a role for *ERα* in cardiac myocyte response to hypertrophic stimuli, however the similarity in phenotype between *ERα* heterozygous and cKO mice suggests a potential contribution of *Cre* expression to the adverse response observed.

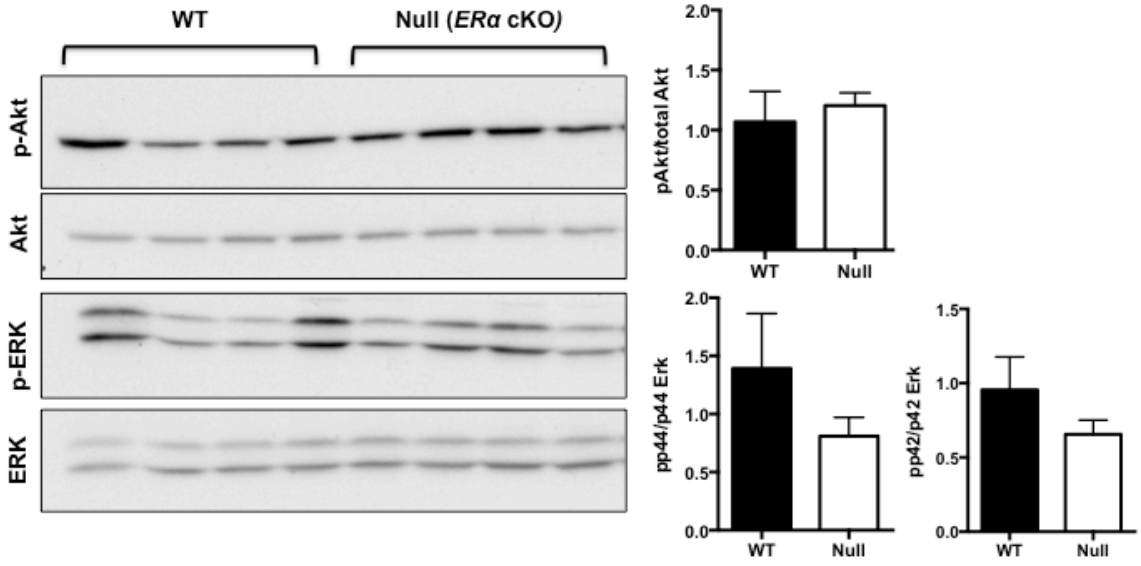

Figure 3.9: Myocardial signaling pathway activation in female control and *ERα* cKO mice. pAkt/PI3K and pERK/MAPK signaling pathway activation were assessed in control, WT and *ERα* cKO mice by immunoblot of left ventricular lysates. Activation of both pathways was unaffected by *ERα* deletion in cardiac myocytes.

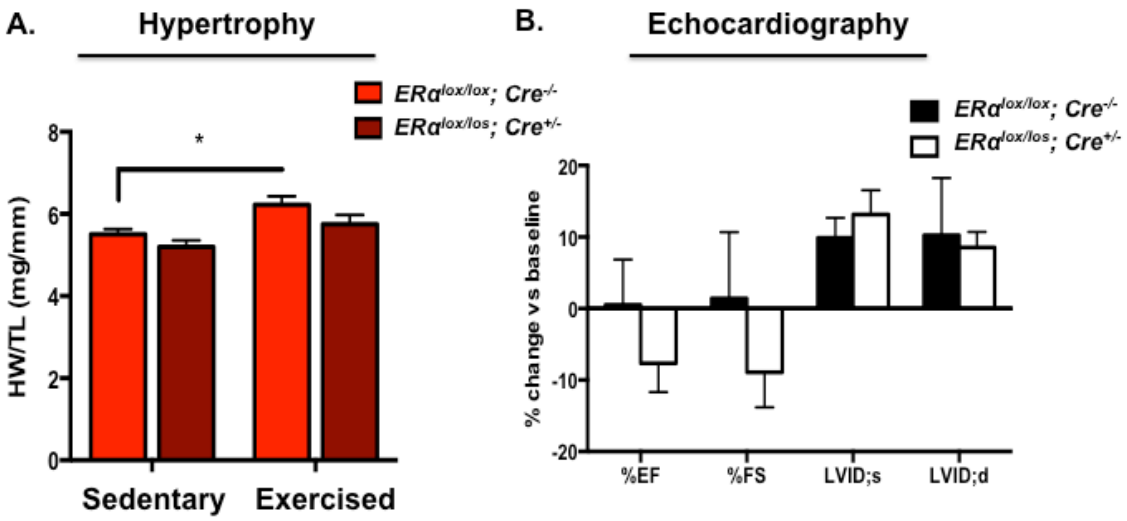

Figure 3.10: Voluntary exercise in female WT and *ERα* cKO mice. WT and *ERα* cKO mice underwent voluntary wheel running (exercise) or were maintained as sedentary controls. Following 21 days, normalized heart size was compared between genotypes and conditions to determine hypertrophy **(A)**. Cardiac function and chamber morphology were measured by echocardiography and compared between the start and end of the exercise program for each genotype **(B)**.

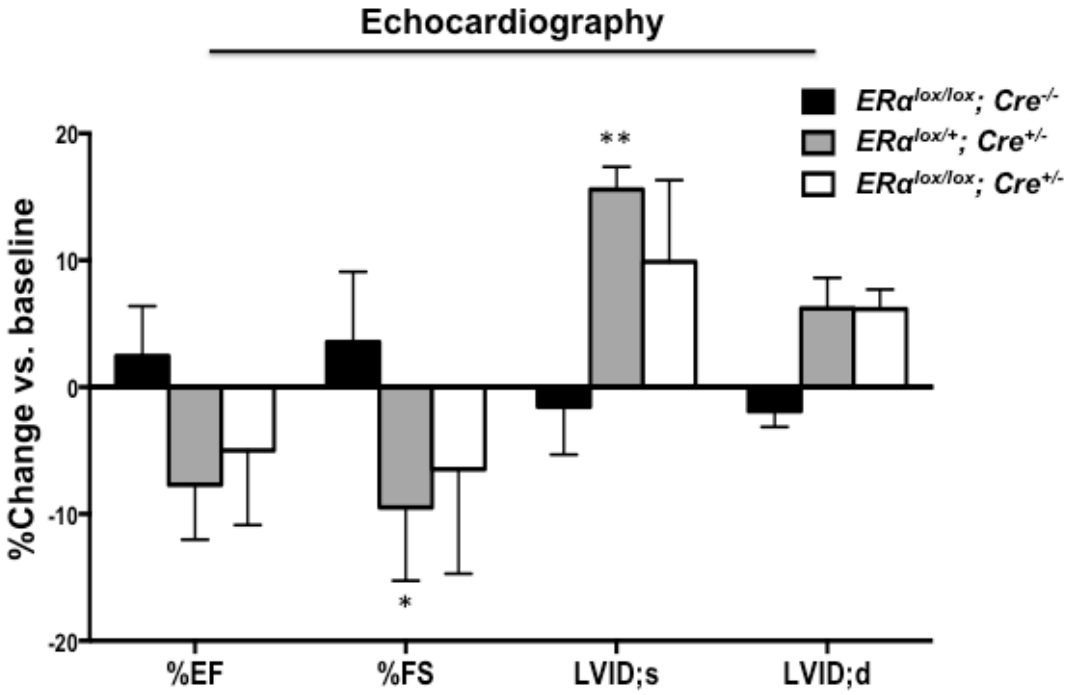

Figure 3.11: Isoproterenol treatment in WT, heterozygous, and *ERα* cKO mice. WT, heterozygous, and *ERα* cKO mice were treated with 30mg/kg/day isoproterenol via osmotic minipump. Cardiac function and morphology were

assessed prior to and following Iso treatment and compared between timepoint and genotypes. %EF: %Ejection fraction, %FS: %Fractional shortenting, LVID;s: Left ventricular internal dimension (systole), LVID;d: Left ventricular internal dimension (diastole). *P<0.05, **P<0.01 vs. WT ($ER\alpha^{lox/lox}$; $Cre^{-/-}$) mice.

3.4 Discussion

3.4.1 Consequences of ERα *deletion in cardiac myocytes: Baseline cardiac biology*

At 3 months of age, conditional deletion of *ERα* in cardiac myocytes had appreciable consequences only at the gene expression level in female mice and to a lesser extent in male mice. Functionally and morphologically, *ERα* cKO mice were otherwise normal at this early time point. These data suggest that in females, *ERα* exerts a considerable beneficial effect in the cardiac myocyte pool. Induction of Natriuretic peptide genes, like *Anp*, and increased expression of Myh7 (*βMyHC*) relative to *Myh6* (*αMyHC)* are hallmarks of pathological cardiac remodeling and their expression in *ERα* cKO mice is therefore suggestive of a protective role for *ERα.*

Longitudinally, it appears *ERα* may be critical for cardiac myocyte maintenance, however data presented in Chapter 4 present a compelling argument that the aging phenotype observed in *ERα* cKO mice is not attributable to loss of *ERα* in the myocytes but rather *Cre* expression in these cells. The gene expression phenotype observed at 3 months in *ERα* cKO mice may be partially attributable to *Cre* expression as well, but in the case of female *ERα* cKO mice, it is slightly more severe than what is observed in *Cre* only mice (described in detail in Chapter 4). In contrast, the pattern observed in male *ERα* cKO mice is slightly attenuated compared to what is observed in male *Cre* only mice (described in detail in Chapter 4) which is again suggestive of a sexually dimorphic influence of both *ERα* and *Cre* expression in cardiac myocytes. Interestingly, *ERα* may

actually be protective against *Cre* related cardiac toxicity in females.

Of note, an analogous genetic model was recently published and supports many of the findings reported in this chapter (Devanathan et al., 2014). In their cardiac myocyte-specific *ERα* model, Devanathan *et a*l. observed mild cardiac morphometric differences between WT and cKO mice as well as modifications in cardiac gene expression. Many of these changes, including induction of *Anp,* were also seen in my cohort of mice (Devanathan et al., 2014). Although the response of their mice to cardiac stressors was not tested, Devanathan *et a*l.'s conclusions generally validate data presented in this chapter.

3.4.2 Consequences of ERα *deletion in cardiac myocytes: Response to cardiac stress*

Female *ERα* cKO mice were unable to achieve normal physiological cardiac hypertrophy following 21 days of wheel running however cardiac function was not adversely affected in these mice (Allen et al., 2001). These results suggest a potential mechanism for *ERα* regulation of physiological cardiac myocyte hypertrophy. In contrast, although Female *ERα* cKO mice achieved significant cardiac hypertrophy following treatment with a pathological stimulus, cardiac function was adversely affected. Thus, the role for *ERα* in cardiac myocyte adaptation to stress differs depending on the nature of the stressor. One potential molecular mediator of this differential requirement for E2/ ERα could be the PI3K/Akt pathway which is required for physiological or beneficial cardiac hypertrophy and can be activated by E2-ERα (McMullen et al., 2007; McMullen et al., 2003; Simoncini et al., 2000).

3.4.3 Conclusions and perspectives

Together these data support the need for further investigation into the molecular mechanism by which E2-ERα can differentially regulate physiological and pathological cardiac adaptation in cardiac myocytes. However, based on data presented in the next chapter, *in vivo* studies must proceed with caution and in conjunction with additional genetic controls. In light of data in Chapter 2, a genome-wide map of cardiac myocyte ERα targets in conjunction with transcriptome data from WT and ERα cKO myocytes would inform on the transcriptional mechanism of cardiac myocyte regulation by this cardiac myocyte-specific estrogen receptor.

Chapter 4

Prolonged Cre expression driven by the α-myosin heavy chain promoter can be cardiotoxic

As published in Pugach, E.K., Richmond, P., Azofeifa, J., Dowell, R., Leinwand L.A. "Prolonged Cre expression driven by the α-Myosin Heavy Chain promoter can be cardiotoxic." *Journal of Molecular and Cellular Cardiology* 2015 Jul 2;86:54-61.

Abstract

Studying the importance of genetic factors in a desired cell type or tissue necessitates the use of precise genetic tools. With the introduction of bacteriophage Cre recombinase/*loxP* mediated DNA editing and promoter-specific *Cre* expression, it is feasible to generate conditional knockout mice in which particular genes are disrupted in a cell type-specific manner *in vivo*. In cardiac myocytes, this is often achieved through α-myosin heavy chain promoter (α*MyHC*)-driven *Cre* expression in conjunction with a *loxP*-site flanked gene of interest. Recent studies in other cell types demonstrate toxicity of *Cre* expression through induction of DNA damage. However, it is unclear to what extent the traditionally used α*MyHC*-*Cre* line (Agah et al., 1997) may exhibit cardiotoxicity. Further, the genotype of α*MyHC*-*Cre*$^{+/-}$ is not often included as a control group in cardiac myocyte-specific knockout studies. Here we present evidence that these α*MyHC*-*Cre*$^{+/-}$ mice show molecular signs of cardiac toxicity by 3 months of age and exhibit decreased cardiac function by 6 months of age compared to wild-type littermates. Hearts from α*MyHC*-*Cre*$^{+/-}$ mice also display evidence of fibrosis, inflammation, and

DNA damage. Interestingly, some of the early functional changes observed in α*MyHC-Cre*$^{+/-}$ mice are sexually dimorphic. Given the high level of Cre recombinase expression resulting from expression from the α*MyHC* promoter, we asked if degenerate *loxP*-like sites naturally exist in the mouse genome and if so, whether they are affected by Cre in the absence of canonical *loxP*-sites. Using a novel bioinformatics search tool, we identified 619 *loxP*-like sites with 4 or less mismatches to the canonical *loxP*-site. 227 sites overlapped with annotated genes and 55 of these genes were expressed in cardiac muscle. Expression of ~26% of the 27 genes tested was disrupted in α*MyHC-Cre*$^{+/-}$ mice indicating potential targeting by Cre. Taken together, these results highlight both the importance of using α*MyHC-Cre* mice as controls in conditional knockout studies as well as the need for a less cardiotoxic *Cre* driver for the field.

4.1 Introduction

When genes were first discovered as the fundamental heritable units the race was on to determine individual gene function. The gold standard for assessing gene function has since been gene deletion, or knockout, and gene overexpression. Increasingly precise genetic engineering techniques enable investigators to perform both of these tasks. Perhaps the most important contributor to this field over the past 30 years has been the introduction of bacteriophage P1 Cre recombinase-mediated recombination of mammalian genomic DNA (reviewed in (Branda and Dymecki, 2004). Hundreds of studies have since utilized this technology to modify a gene of interest at the cell or organismal level in an effort to study its requirement or sufficiency (Figure 4.1).

By expressing Cre in a tissue or cell-type specific manner, genes that are engineered with flanking *loxP* sites can be deleted or overexpressed in an analogous

fashion. In the adult cardiac myocyte, this is most often achieved using *Cre* expression driven by the cardiac myocyte-specific α-*myosin heavy chain* (α*MyHC*) promoter/enhancer (Agah et al., 1997; Subramaniam et al., 1991). *Cre* expression from this promoter has been shown to be both cardiac myocyte-specific and to drive highly efficient recombination (Agah et al., 1997). Use of this transgenic mouse circumvents embryonic or perinatal lethality induced by deletion of genes required for other cell types and has therefore uncovered many cardiac myocyte-specific genetic roles. To date, nearly 130 primary research studies (Appendix II) have utilized this transgenic mouse to conditionally modify a gene of interest in cardiac myocytes. Thus, this tool has become indispensible to the cardiac biology field.

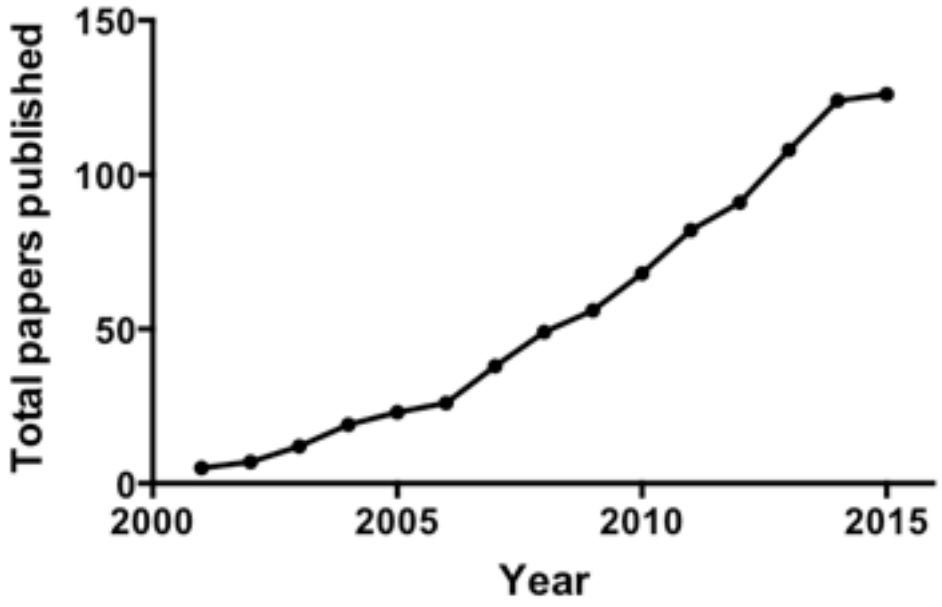

Figure 4.1: To-date publications utilizing Agah *et al.* α*MyHC-Cre* mice.

Cre recombinase mediates recombination between pairs of 34-base-pair palindromic *loxP* sites (Sternberg et al., 1981). Although its preference is for sites consisting of two 13-base-pair inverted repeats separated by an 8-base-pair spacer (Hoess

et al., 1986), Cre has been shown to have promiscuous activity and is capable of recombination at sites containing up to 10 mismatches to the canonical *loxP* site (Thyagarajan et al., 2000). Such degenerate *loxP* sites have been identified in mammalian genomes and can serve as legitimate Cre substrates (Ito et al., 2010). Further, it has been shown that Cre binding to *loxP* sites in the absence of recombination has the ability to block downstream transcription (Iovino et al., 2005). Thus, Cre has the potential for a variety of off-target effects.

Indeed, several instances of off-target Cre effects have been documented over the past decade. In non-cardiac tissue cell-types, Cre expression has been shown to induce DNA damage and apoptosis in the absence of *bona fide loxP* sites (Janbandhu et al., 2013; Schmidt et al., 2000). Toxic effects associated with Cre expression have been observed in gastrointestinal cells, neurons, and spermatids (Harno et al., 2013; Jae Huh et al., 2010; Schmidt et al., 2000). Finally, tamoxifen-inducible Cre expression in myocardium has also been demonstrated to induce fibrosis, DNA damage, and cardiac dilation independently of tamoxifen-associated toxicity (Bersell et al., 2013; Buerger et al., 2006; Koitabashi et al., 2009; Lexow et al., 2013). However a *Cre*-only control genotype is not often included in studies requiring Cre expression. In fact, the α*MyHC-Cre*$^{+/-}$ genotype is only included as a control group in ~20% of studies identified that utilize this *Cre* line (Appendix II and Figure 4.2).

Given the extensive use of the α*MyHC-Cre* mice and anecdotal reports of associated cardiotoxicty (Davis et al., 2012; Doetschman and Azhar, 2012; Molkentin and Robbins, 2009), we sought to formally assess whether cardiac myocyte-restricted Cre expression in the absence of engineered *loxP* sites modified cardiac biology. We

hypothesized that prolonged myocardial Cre expression would lead to cardiotoxicty. To test our hypothesis, we took a combined functional, molecular, and bioinformatics approach. We examined a panel of genes whose expression consistently changes with cardiac pathology and discovered significant changes in α*MyHC-Cre*$^{+/-}$ mice at 3 months of age that progressed to more profound changes by 6 months of age. These molecular changes were accompanied by a decrease in cardiac function in these mice as well as pathological signaling pathway activation and evidence of an activated DNA damage response. We suggest that during prolonged Cre expression, endogenous, non-canonical *loxP* sites are targeted, thus activating a DNA damage response that is associated with pathological signaling pathway and gene expression activation, and adverse remodeling of the myocardium.

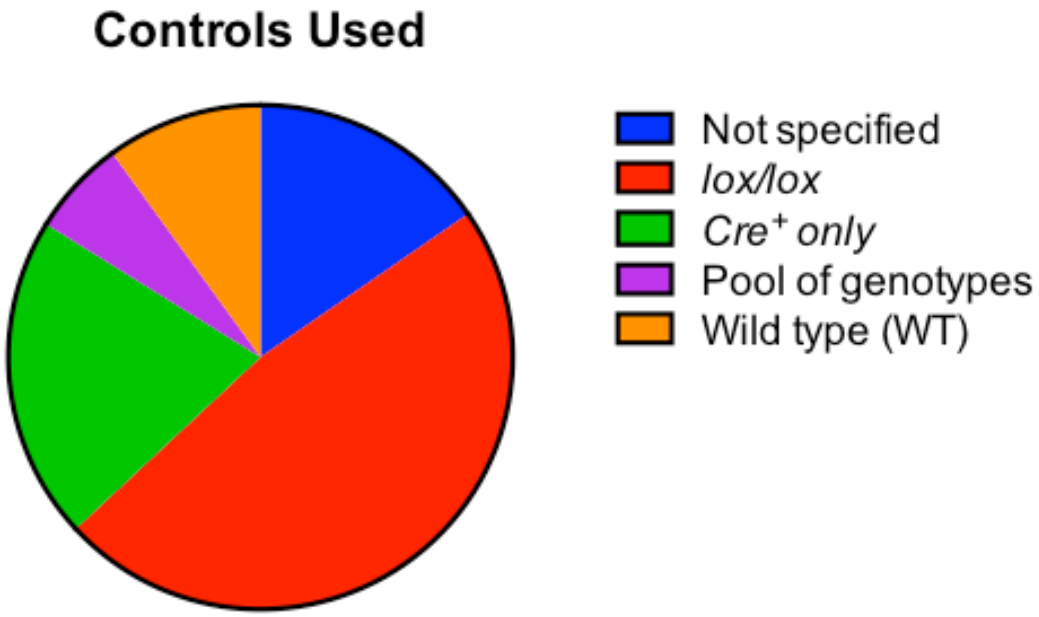

Figure 4.2: Published usage of Agah *et al.* α*MyHC-Cre* mice and specified control genotypes from 1997-2015. N=128 paper, 18 patents, and 12 dissertation.

4.2 Materials and methods

Mice

All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Colorado at Boulder. Mice were fed *ad libitum* standard rodent chow and housed in a facility with a 12 hour light, 12 hour dark cycle. α*MyHC-Cre* mice were backcrossed into the C57Bl/6J background for at least 10 generations and bred using heterozygote males and wild-type (WT) females. For all experiments, WT mice were compared to α*MyHC-Cre*$^{+/-}$ mice. Genotyping was performed from tail snip biopsies and re-confirmed post-mortem. Primers for genotyping are listed in Appendix I. Myocardial *Cre* expression was confirmed by quantitative real-time reverse transcriptase PCR (qRT-PCR) and western blot for Cre expression (Figure 4.3). For sample collection, animals were sedated using 1–4% inhaled isoflurane and sacrificed by cervical dislocation. Hearts were excised, perfused in ice-cold PBS, and flash frozen in liquid nitrogen.

Transthoracic echocardiography

Non-invasive echocardiographic images and measurements were made using the Philips Sonos 5500 system. Mice were placed on a heating pad and maintained on 2% isoflurane via spontaneous inhalation. The mouse's fur was first removed from the ribcage using a depilatory cream, and an image-potentiating gel was then applied for image acquisition. M-(motion) mode images were captured for each animal at the level of the papillary muscles (A2 view). Left ventricular dimensions and function were calculated from the M-mode images, where wall thickness of the anterior and posterior walls and the chamber diameter were measured using the ASE leading edge convention.

Gene expression

Total RNA was purified using TRI Reagent (Ambion) according to the manufacturer's protocol. cDNA was synthesized with Superscript III reverse transcriptase (Invitrogen) and random hexamer primers. Gene expression was determined by qRT-PCR using SYBR Green dye with gene specific primer sets (Appendix I) and a Bio-Rad CFX-96 Real-Time PCR system.

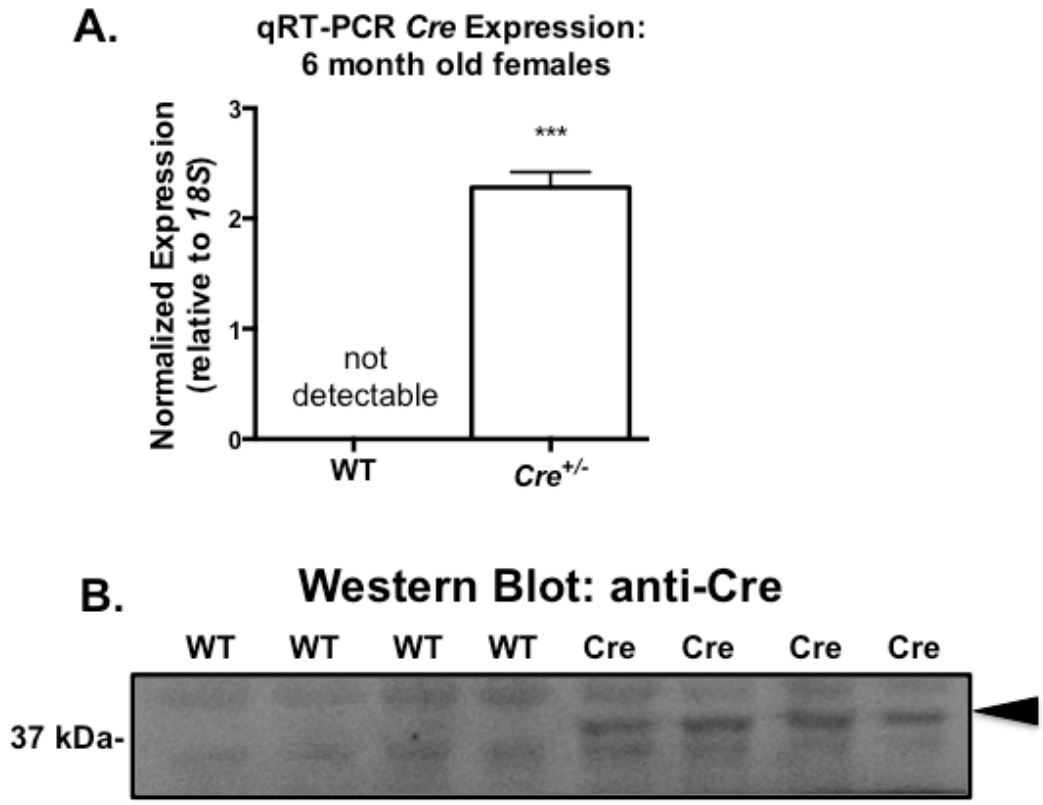

Figure 4.3: *αMyHC-Cre* ventricular mRNA and protein expression. *Cre* mRNA expression by qRT-PCR **(A)** and Cre protein expression by immunoblot **(B)**. Error bars: SEM, N=4-6/group ***P<0.001 vs. WT control.

Western blot

Left ventricles were homogenized in 50mM Tris pH 8.0, 150mM NaCl, 1% NP40, 0.5% Na-deoxycholate, 0.1% SDS, complete protease inhibitor cocktail (Roche) and the

following phosphatase inhibitors: 1mM PMSF, 2mM NaF, 2mM NaPPi, 1mM Beta-Glycerophosphate, 1mM Na-molybdate dihydrate, and 1mM Na-Orthovanadate. 10µg of protein was resolved on an SDS-PAGE gel and probed with the following antibodies: GAPDH (Cell Signaling Technologies 2118s), Cre (Cell Signaling Technologies 7803), phospho-ERK (Cell Signaling Technologies 9101s), total ERK (Cell Signaling Technologies 9102s), phospho-p38 (Cell Signaling Technologies 4511s), total p38 (Cell Signaling Technologies 9212s), MCIP1.4 (Bush et al., 2004), Calcineurin (CnA) (Cell Signaling Technologies 2614), γH2AX (Cell Signaling Technologies 9718), p53 (Santa Cruz 6243), Bax (Santa Cruz 526), PARP (Cell Signaling Technologies 9542), and ME3 (Novus NBP1-30525).

Identification of degenerate loxP *sites*

To identify putative, degenerate *loxP* sites we used LADMA (Levenshtein Automata based Degenerate Motif Annotator) to annotate the *Mus musculus* genome (http://hgdownload.cse.ucsc.edu/goldenPath/mm10/bigZips/), allowing up to 4 mismatches based on a conservative input Cre binding motif sequence: ATNACNNCNTATA NNNTANNN TATANGNNGTNAT (Thyagarajan et al., 2000) (http://dowell.colorado.edu/resources.html). Briefly, the Levenshtein distance is defined by the minimal number of insertions, deletions or substitutions required to transform one string to another (Schulz and Mihov, 2002). Thus, LADMA accepts a number of mismatches (in our case 4) and builds a Levenshtein non-deterministic finite state automata (NFA) to find all possible DNA subsequences within some Levenshtein distance. To increase computational efficiency, this NFA is converted to a deterministic finite state automata and inserted into an Aho-Corasick-like data structure in order to find

potential matches across the entire genome (Aho and Corasick, 1975).

LADMA was run on each chromosome and returned 617 total sites finding 0 perfect match, 0 1-mismatch, 12 2-mismatches, 244 3-mismatches, and 361 4-mismatches (Appendix III). These sites were then intersected with full-length gene bodies extracted from UCSC mm10 Ensembl annotations with the introns included (http://hgdownload.cse.ucsc.edu/goldenPath/mm10/database/) using BedTools IntersectBed (BedTools version 2.16.2) (Quinlan and Hall, 2010). This resulted in 227 genes that contained one or more degenerate binding site(s).

Transgene copy number analysis

Transgene copy number was estimated as described (Joshi et al., 2008). Briefly, genomic DNA (gDNA) was isolated using phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. gDNA was then digested overnight with EcoRI (New England Biolabs). Purified, digested DNA was quantified and 10 ng were loaded into a qPCR reaction. Standard curves were constructed using known copy numbers of linearized plasmids containing the coding region of *Cre* or *Myh15*. Copy number was normalized to *Myh15* (2 copies/genome).

Determination of Cre *transgene insertion site*

To determine insertion site of the α*MyHC-Cre* transgene, Thermal Asymmetric Interlaced PCR (TAIL-PCR) was performed as described (Liu and Whittier, 1995; Pillai et al., 2007). Briefly, we performed three rounds of nested PCR on purified gDNA in which one primer was anchored in the 3' end of the α*MyHC-Cre* transgene and degenerate oligonucleotides were used as reverse primers. PCR products were purified, sequenced, and mapped to the mouse genome.

Histology

Hearts were fixed in 10% buffered formalin overnight and then transferred to 70% ethanol. Fixed hearts were embedded in paraffin, sectioned, and stained with Picrosirius Red (Polysciences). The Picrosirius Red positive area was quantified as a fraction of total tissue area using ImageJ using either brightfield microscopy or polarized light microscopy (yellow, type I collagen fibers were quantified). 3-4 images were quantified per heart in 3-4 hearts/group. Tissue edges and vessels were excluded from analysis. To identify inflammatory cells, F4/80 immunostaining (Serotec MCA497) was performed to label marcrophages. F4/80 positive cells were counted across transverse sections in 3-4 hearts per genotype. Analogous quantification of TUNEL-positive cells (Roche 11684809910) was also performed in transverse sections to identify DNA fragmentation and/or apoptosis.

Data and statistical analysis

Data are presented as mean ± SEM. Differences between groups were evaluated for statistical significance using Student's two-tailed t test (two groups) or one-way ANOVA (more than two groups) followed by Tukey's post-hoc test for pairwise comparisons. *P* values less than 0.05 were considered significant unless otherwise noted.

4.3 Results

4.3.1 αMyHC-Cre expression is cardiotoxic in an age-dependent manner

Although a cardiotoxic effect of myocardial *Cre* expression in the widely used α*MyHC-Cre* mice has been reported (Davis et al., 2012; Doetschman and Azhar, 2012; Molkentin and Robbins, 2009), to our knowledge no thorough investigation of this phenotype has been published. To that end, we chose to formally characterize the effect

of myocardial *Cre* expression driven by the 5.5 kb murine α*MyHC* promoter (Agah et al., 1997). We characterized these mice at 3 months of age as this has been a commonly used adult time-point in a literature search of studies employing this transgenic line (Appendix II). Further, we examined males and females separately as it is known that the heart is a sexually dimorphic organ yet many studies in which this line had been utilized did not specify sex (Luczak and Leinwand, 2009). At 3 months, no gross abnormalities were observed in systolic cardiac function (%Ejection Fraction, %EF) in female α*MyHC-Cre*$^{+/-}$ mice as compared to wild-type (WT) littermates (Figure 4.4A,B and Table 4.1). In males, however, we observed an increase in cardiac function and an increase in heart rate (HR) that coincided with a trend towards hypertrophy in α*MyHC-Cre*$^{+/-}$ males (Figure 4.4B and Table 4.1). Although the increase in HR may mediate the observed change in cardiac function (Higgins et al., 1973; Schaefer et al., 1988), we suspect these changes may be due to a compensatory state preceding functional decline. Further, we observed a correlative relationship between *Cre* expression and cardiac function at this time point suggesting a dose-dependent effect of myocardial *Cre* expression (Figure 4.5A-C).

We next examined gene expression in the ventricles of 3 month-old mice since such molecular changes frequently precede functional changes (Villarreal and Dillmann, 1992). To that end, we examined reactivation of fetal gene expression of naturietic peptides, *Anp* and *Bnp*, as well as selective cardiac myosin expression in hearts of male and female mice. We observed statistically significant, though modest, increases in *Anp* and *Bnp* in both males and females but did not observe changes in any other genes (Figure 4.4C).

Table 4.1: Morphometric and functional M-mode echocardiography. Summary of echocardiography measurements made in 3 and 6 month old male and female mice. N/group is displayed in column heading $*P < 0.05$, $**P < 0.01$ vs. WT, age-matched control.

3 months

(N)	WT Female (6)	*Cre*$^{+/-}$ Female (14)	WT Male (6)	*Cre*$^{+/-}$ Male (10)
%EF	59.57 ± 2.13	62.92 ± 1.41	54.55 ± 1.90	**64.24 ± 1.72
LVID;s (cm)	0.270 ± 0.0057	0.258 ± 0.00283	0.294 ± 0.00835	*0.259 ± 0.00618
LVID;d (cm)	0.388 ± 0.00471	0.384 ± 0.00355	0.408 ± 0.00991	0.408 ± 0.00844
LVAW;s (cm)	0.12 ± 0.0032	0.12 ± 0.0023	0.12 ± 0.0043	0.13 ± 0.0033
LVAW;d (cm)	0.062 ± 0.0018	0.058 ± 0.0010	0.066 ± 0.0015	0.066 ± 0.0012
LVPW;s (cm)	0.0983 ± 0.00320	0.102 ± 0.00156	0.102 ± 0.00198	*0.109 ± 0.0014
LVPW;d (cm)	0.062 ± 0.0014	0.061 ± 0.00074	0.067 ± 0.0010	0.067 ± 0.00090
Heart Rate (bpm)	434 ± 10.3	443 ± 12.7	439 ± 13.8	**503 ± 9.3
LV Vol;s	26.36 ± 1.40	23.56 ± 0.77	33.65 ± 2.26	26.78 ± 2.46
LV Vol;d	65.32 ± 1.87	63.92 ± 1.42	74.09 ± 4.29	74.08 ± 3.73
LV mass	64.22 ± 1.21	*58.57 ± 1.45	77.26 ± 4.44	76.39 ± 2.09

6 months

(N)	WT Female (4)	*Cre*$^{+/-}$ Female (6)	WT Male (4)	*Cre*$^{+/-}$ Male (8)
%EF	68.41 ± 2.70	**57.80 ± 1.67	65.07 ± 1.60	*57.24 ± 1.72
LVID;s (cm)	0.232 ± 0.0117	**0.275 ± 0.00693	0.256 ± 0.0100	0.276 ± 0.0118
LVID;d (cm)	0.360 ± 0.00466	**0.397 ± 0.0029	0.395 ± 0.0123	0.397 ± 0.0110
LVAW;s (cm)	0.12 ± 0.0033	0.12 ± 0.0028	0.13 ± 0.0026	0.12 ± 0.0022
LVAW;d (cm)	0.059 ± 0.0	***0.067 ± 0.0010	0.068 ± 0.0	0.068 ± 0.00037
LVPW;s (cm)	0.106 ± 0.0052	0.104 ± 0.0038	0.109 ± 0.0016	0.107 ± 0.0032
LVPW;d (cm)	0.059 ± 0.0	*0.067 ± 0.0016	0.068 ± 0.0016	0.067 ± 0.00037
Heart Rate (bpm)	531 ± 8.8	506 ± 9.6	466 ± 30.7	492 ± 18.9
LV Vol;s	18.82 ± 2.41	**28.54 ± 1.67	23.97 ± 2.31	29.27 ± 2.88
LV Vol;d	59.45 ± 4.98	67.39 ± 1.76	68.41 ± 4.88	69.56 ± 4.49
LV mass	57.36 ± 3.00	**72.40 ± 2.53	74.78 ± 2.91	75.29 ± 3.48

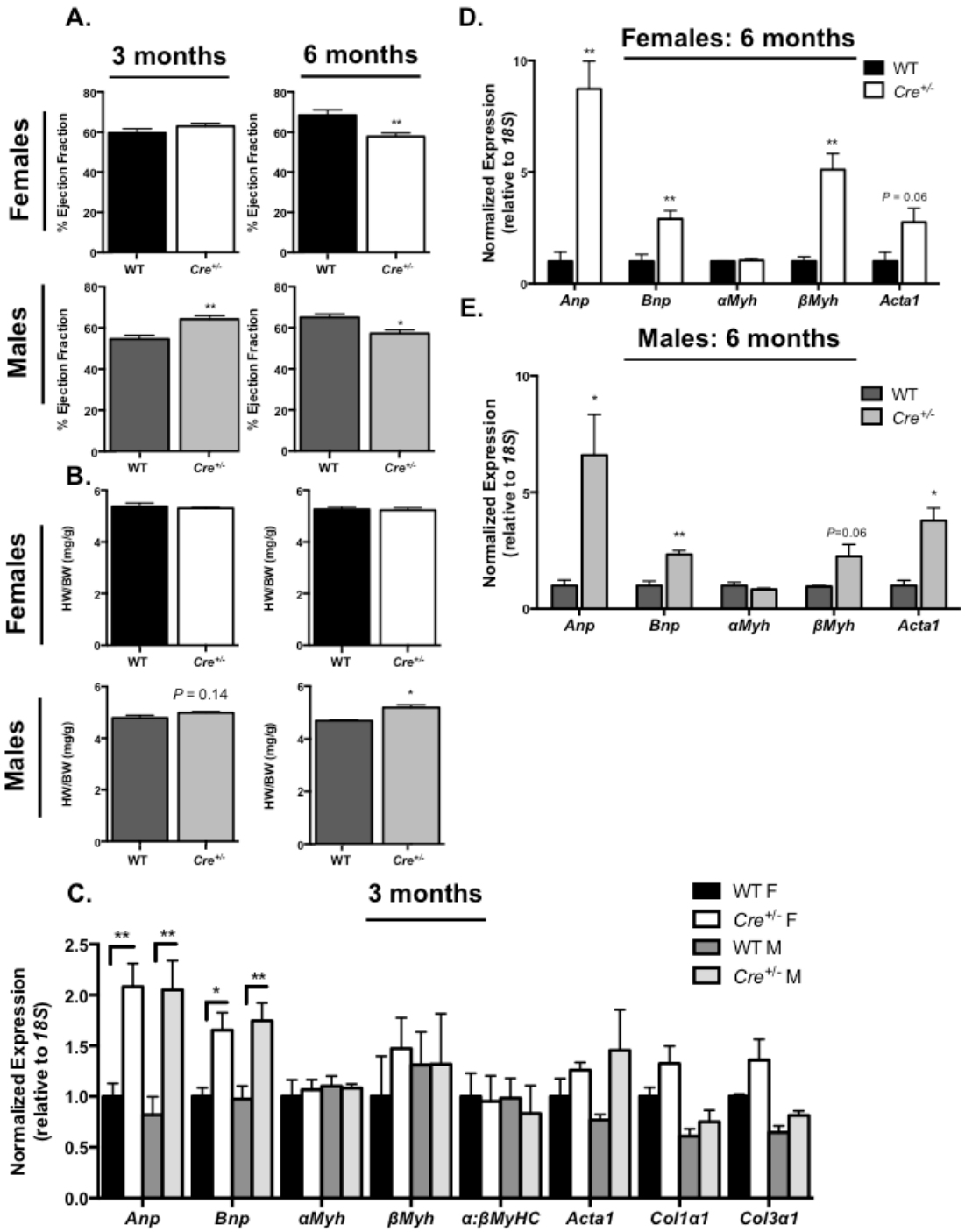

Figure 4.4: *αMyHC-Cre* is cardiotoxic by six months of age. (A) Ventricular contractile function (% Ejection fraction) at 3 months and 6 months. **(B)** Cardiac mass, heart weight normalized to body weight (HW/BW) at indicated ages. **(C)** Cardiac gene expression at 3 months in males and females **(C)** and 6 months, females **(D)**, and males **(E)**. Error bars: SEM, N=4-6/group **P* < 0.05, ***P* < 0.01 vs. age-matched and sex-matched WT control.

In light of the observed changes in gene expression observed at 3 months, we next asked whether there was a resultant decline in cardiac function at 6 months in α*MyHC-Cre*$^{+/-}$ mice. Indeed, cardiac function was significantly decreased at 6 months in both males and females (Figure 4.4A) and significant hypertrophy was observed in males as compared to WT littermates (Figure 4.4B). Further, expression of fetal genes was increased and myosin expression was shifted toward a pathological state (elevated *βMyHC*) at this time-point (Figure 4.4D,E) thus supporting our initial hypothesis that the gene expression changes and hypertrophy observed at 3 months were likely precursors to the more notable changes observed at 6 months. We therefore conclude that α*MyHC-Cre* expression in this model is cardiotoxic in an age-dependent manner.

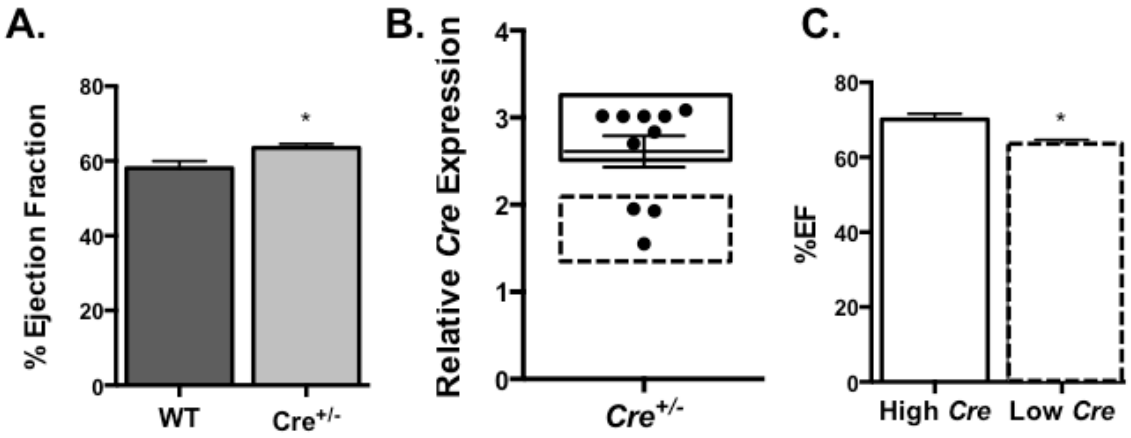

Figure 4.5: Cre expression correlates with cardiac function at 3 months. (A). %EF in 3 month-old mice. **(B)** *Cre* expression by qRT-PCR. *Cre* expression is grouped into "high" (solid line) and "low" (dashed line). **(C)** %EF separated into *Cre* "high" and *Cre* "low" demonstrates dose-dependence of *Cre* effect on %EF. N=4-6/group *$P < 0.05$.

In order to better characterize the observed cardiotoxicity at 6 months, we examined pathological signaling pathway activation. In agreement with the changes in gene expression and cardiac function, we observed activation of the stress-induced,

pathological p38 MAPK signaling pathway (Figure 4.6A). We did not observe activation of pro-hypertrophic P-ERK signaling but other pathological and stress induced signaling molecules such as MCIP1.4 and Calcineurin (CnA) were also upregulated in α*MyHC-Cre*$^{+/-}$ mice (Figure 4.6A).

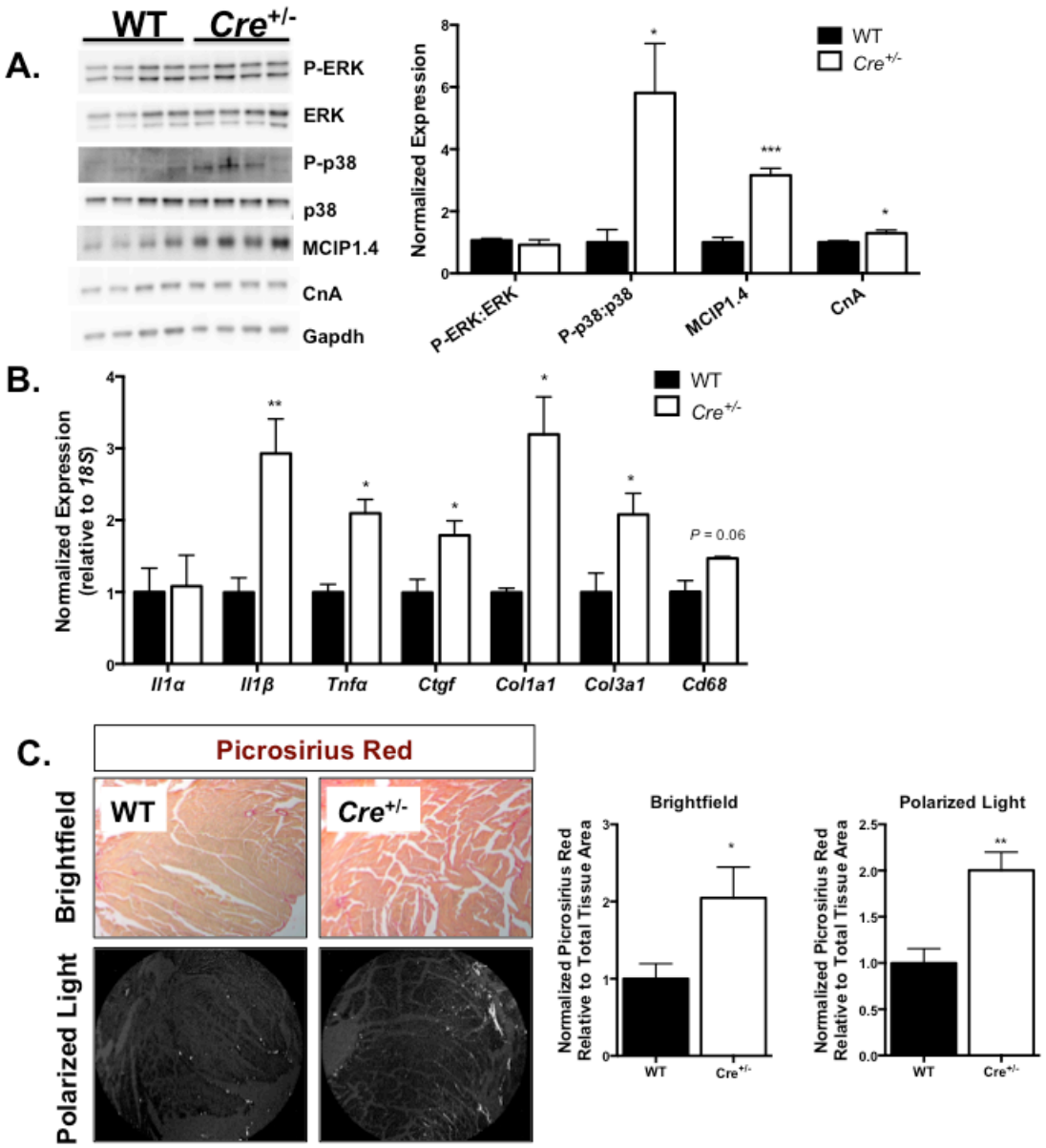

Figure 4.6: Induction of pathological intracellular signaling and inflammatory or fibrotic genes in female α*MyHC Cre*$^{+/-}$ ventricles at 6 months (A) MAPK phosphorylation status or MCIP1.4 and Calcineurin protein expression (immunoblot). **(B)** Inflammatory cytokine and fibrotic gene expression (qRT-PCR). **(C)** Histochemical assessment of fibrosis

(Picrosirius Red stain) 10X magnification using either brightfield or polarized light microscopy. Picrosirius Red-positive area was quantified as a percentage of total tissue area. Error bars: SEM, N=4-6/group $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. age-matched and sex-matched WT control.

4.3.2 Prolonged αMyHC-Cre expression is associated with mild fibrosis and inflammation

We then asked whether gene expression related to p38 signaling and cardiac pathology was correspondingly modified. In ventricles from female α*MyHC-Cre*$^{+/-}$ mice we observed increases in several pro-inflammatory markers including *Il-1*β and *Tnf*α, as well as increases in pro-fibrotic makers, *Col1*α*1*, *Col3*α*1*, and *Ctgf* (Figure 4.6B). Further we observed a trending increase in expression of a marker of activated, infiltrating macrophages, *Cd68* (Figure 4.6B). These pro-inflammatory, pro-fibrotic gene expression changes correlated with modest increases (2-fold) in left-ventricular fibrosis (Figure 4.6C) similar to that reported in models of hypertrophic cardiomyopathy and trans-aortic banding (Barrick et al., 2009; Konhilas et al., 2006). A trending increase in inflammatory cell presence was observed in α*MyHC-Cre*$^{+/-}$ ventricular myocardium (Figure 4.7). Comparable changes in gene expression were also confirmed in 6 month-old male α*MyHC-Cre*$^{+/-}$ mice (Figure 4.8). These pro-fibrotic gene expression changes may be directly attributable to activated p38 MAPK signaling (reviewed in (Clerk and Sugden, 2006).

4.3.3 Prolonged αMyHC-Cre expression is associated with DNA damage response

Because Cre is a DNA recombinase, we hypothesized that the observed cardiotoxicity in α*MyHC-Cre*$^{+/-}$ mice may be attributable to Cre-related DNA damage. To this end, we examined DNA damage response effectors in female α*MyHC-Cre*$^{+/-}$ mice

at 6 months of age. Indeed, we saw modest increases in several factors related to DNA damage and apoptosis including cleaved PARP, γH2A.X, p53, and Bax (Figure 4.9A). These changes were accompanied by detectable transcriptional upregulation of master DNA damage response effectors, p21 and p53 in both female and male α*MyHC-Cre*$^{+/-}$ mice (Figure 4.8 and Figure 4.9B) as well as a ~3-fold increase in cells with DNA fragmentation as identified by TUNEL-positive cells (Figure 4.9C).

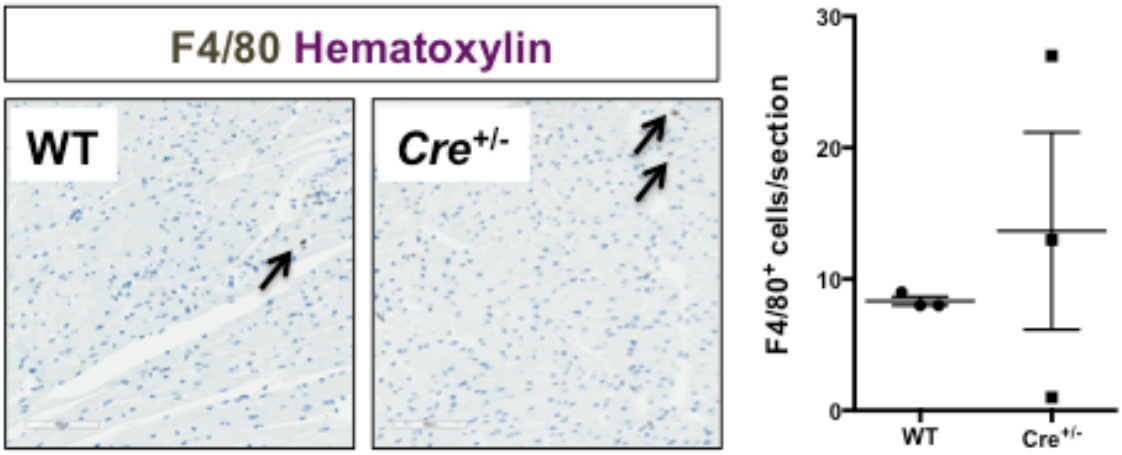

Figure 4.7: Myocardial analysis of inflammatory cells. Histochemical assessment of activated macrophages (F4/80^{+} cells) in ventricular myocardium. Arrows indicate F4/80^{+} cells. Scale bar: 100μm.

4.3.4 Endogenous, degenerate loxP sites may be targeted by Cre following persistent expression under the αMyHC promoter

Given the absence of canonical *loxP* sites in the α*MyHC-Cre*$^{+/-}$ mice in our studies and the presence of a general DNA damage response, we next asked whether this response might be a result of off-target Cre action at degenerate, endogenous *loxP* sites. Using an unbiased bioinformatics approach and a novel motif-finding algorithm (see Materials and Methods), we searched the mouse genome for "*loxP*-like" sites, tolerating mismatches to the canonical *loxP* site at positions known to be dispensable for Cre

binding and recombination (Figure 4.10 (Guo et al., 1997; Thyagarajan et al., 2000).

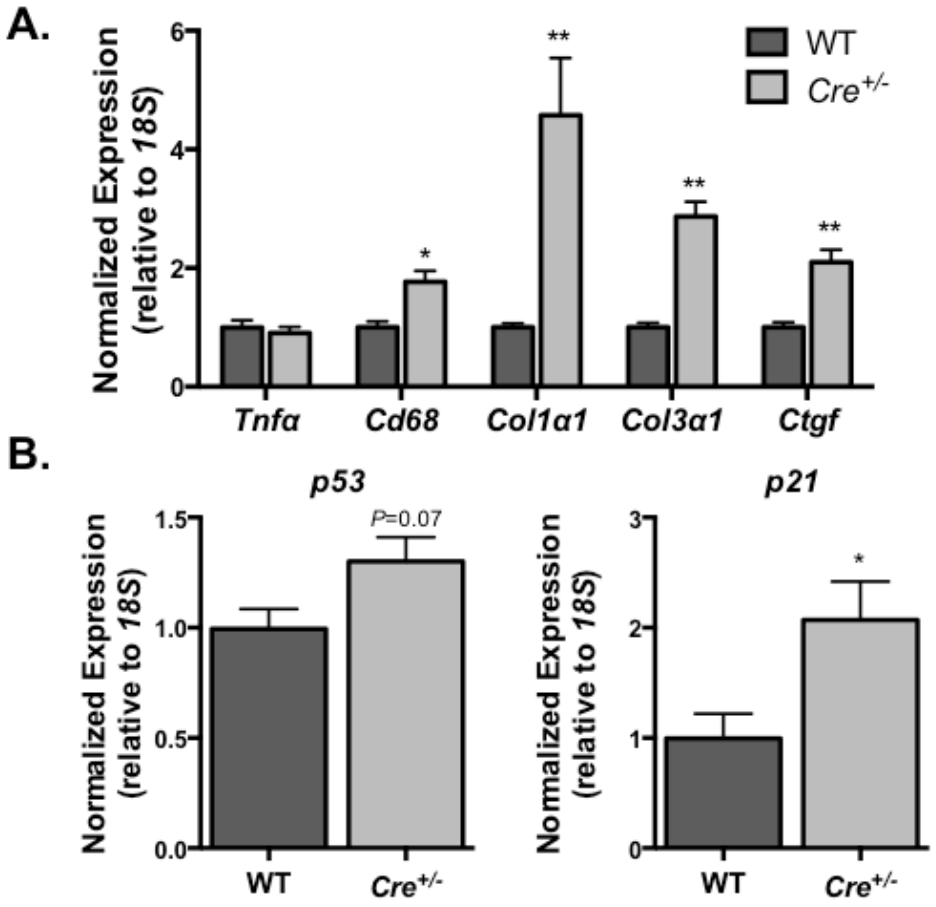

Figure 4.8: Ventricular gene expression changes in 6 month-old α*MyHC-Cre*$^{+/-}$ males. Many of the gene expression changes observed in α*MyHC-Cre*$^{+/-}$ females were confirmed in α*MyHC-Cre*$^{+/-}$ males. Error bars: SEM, N=4-5/group, **P* < 0.05, ***P* < 0.01 vs. age-matched and sex-matched WT controls.

Several hundred of these sites were identified, depending on the number of mismatches to the canonical site tolerated (Appendix III). Further, a large percentage of these sites in the mouse genome (37%) were located intragenically and many (24%) of these were present in transcriptionally active cardiac genes as determined by transcriptome profiling of ventricular myocardium (Matkovich et al., 2010). We next tested whether expression of

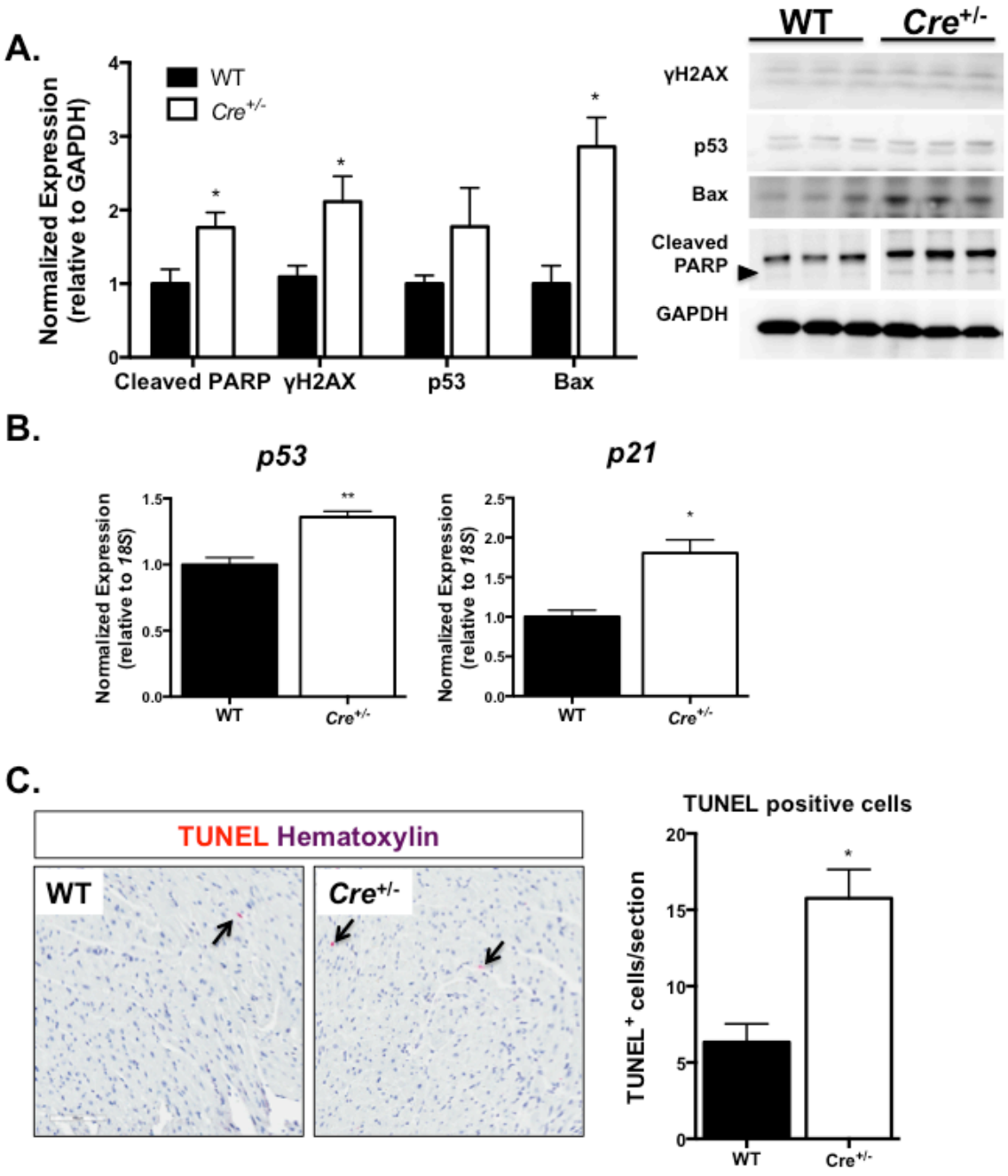

Figure 4.9: Ventricular DNA damage response in 6-month-old female *αMyHC-Cre*$^{+/-}$ mice (A) mRNA expression of DNA damage response regulators, *p21* and *p53* (qRT-PCR). **(B)** DNA damage-related and pro-apoptotic protein levels (immunoblot). **(C)** Histochemical assessment of TUNEL^{+} cells in ventricular myocardium. Arrows indicate TUNEL^{+} cells. Error bars: SEM, N=4-6/group *$P < 0.05$, **$P < 0.01$ vs. age-matched and sex-matched WT control.

27 of these cardiac genes containing degenerate *loxP* sites was modified by the presence of α*MyHC-Cre* by designing qRT-PCR primers downstream of or spanning the putative *loxP* site. We found expression changes for 27% of the genes tested (Figure 4.11A) and confirmed a change in protein expression consistent with the observed change in transcript expression for one target, *ME3* (Figure 4.11B).

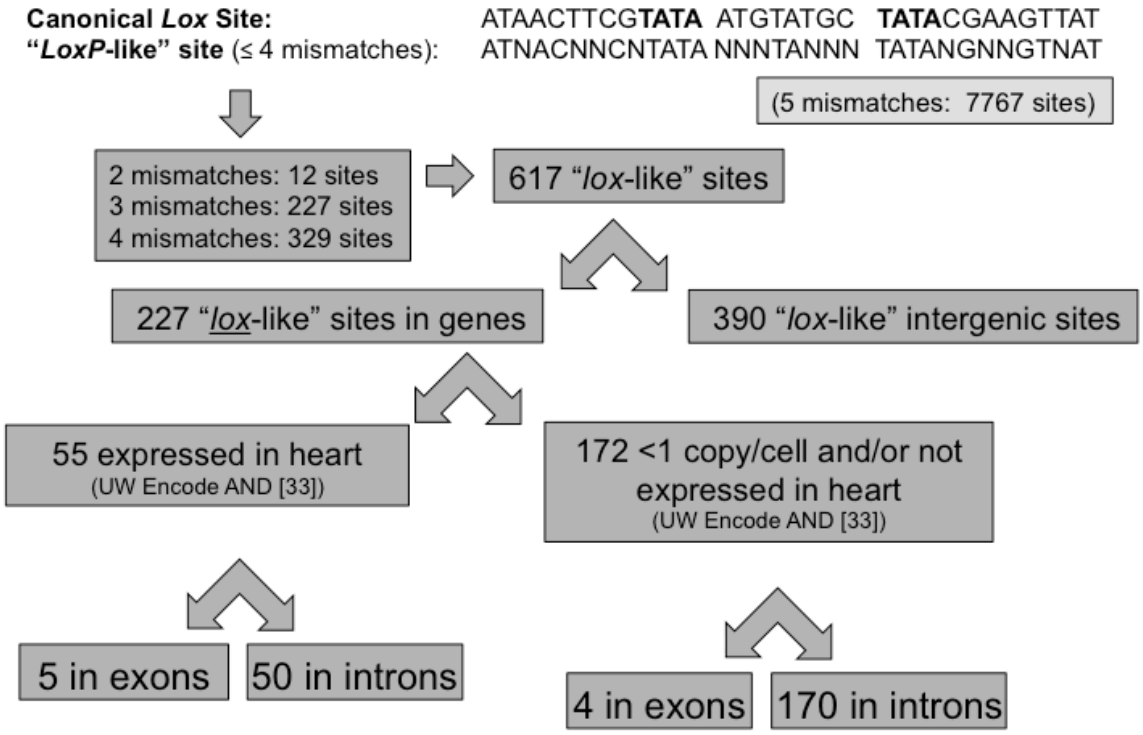

Figure 4.10: Degenerate *loxP* sites are abundant in the mouse genome. Flow chart of genome-wide search for degenerate *loxP* sites in mouse. Degenerate *loxP* site sequence was based on published Cre binding and recombination data (Guo et al. 1997, Thyagarajan et al. 2000). This sequence is a conservative estimate for maximum-tolerated mismatches by Cre. Note that a search for *loxP* sites harboring just two mismatches identified 12 endogenous sites while a search for *loxP* sites harboring five mismatches identified more than 7500 endogenous sites. Transcriptionally relevant genes for the heart were identified by intersecting the list of *loxP*-like containing genes with both the publically available University of Washington (UW) ENCODE dataset and transcriptome data from (Matkovich et al. 2010). Approximately 20% of papers cite α*MyHC-Cre* mice as controls while nearly 50% cite floxed littermates as controls. WT: Wild type, fl/fl: Gene of interest with flanking *loxP* sites.

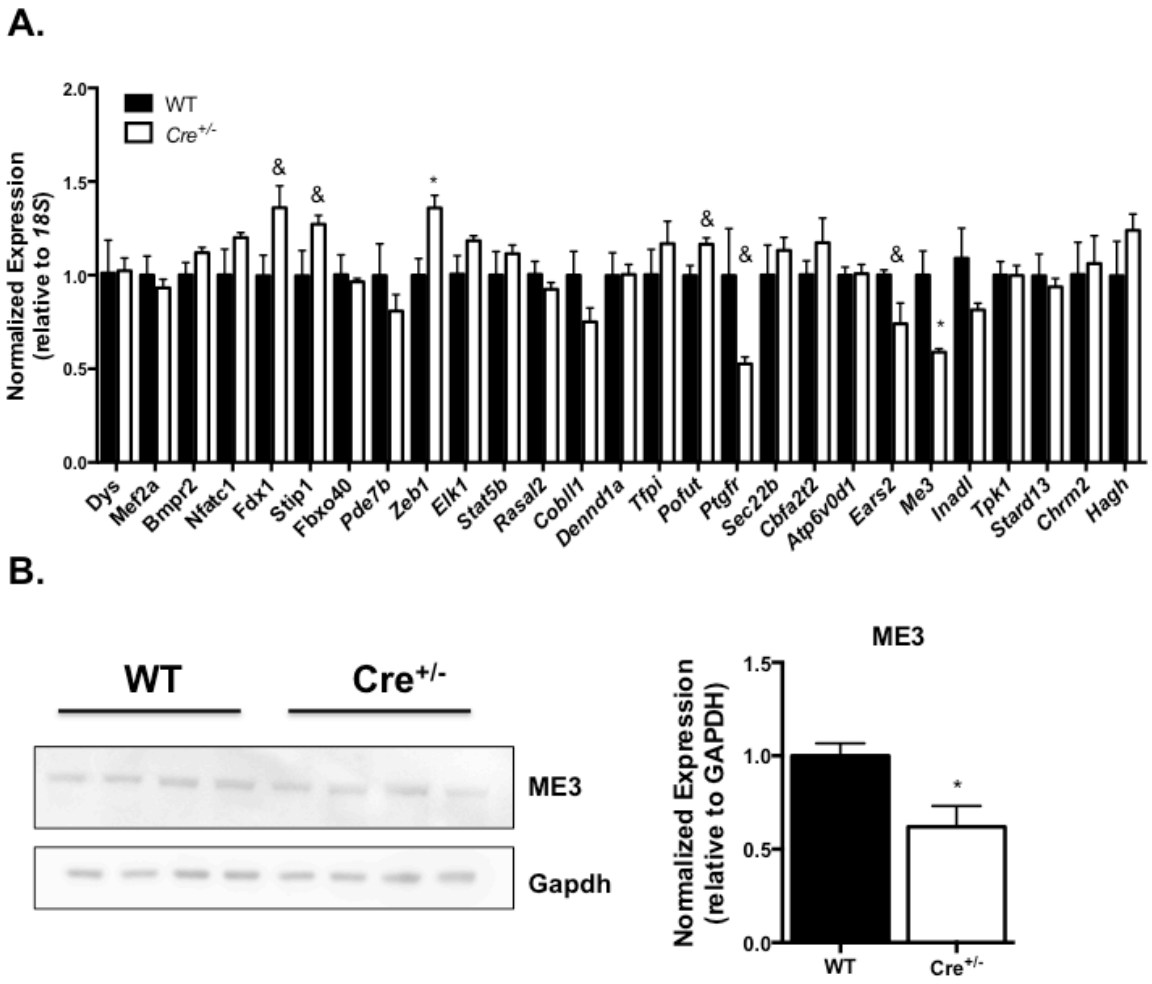

Figure 4.11: Gene expression is disrupted in a subset of genes harboring degenerate *loxP* sites. (A) qRT-PCR mRNA expression in 6 month old α*MyHC-Cre*$^{+/-}$ females of a subset of genes harboring degenerate *loxP* sites. 27/55 (49%) genes identified were tested. Expression changes were observed for 7/27 (26%) of genes tested. **(B)** Protein expression (immunoblot) for ME3 normalized to Gapdh. N=3-4/group *$P < 0.05$ & $P < 0.1$ vs. WT control.

4.3.5 Transgene copy number or insertion site does not likely account for observed cardiotoxicity in αMyHC-Cre mice

Finally, we sought to rule out an effect of cardiotoxicity mediated by transgene insertion site or exceedingly high copy number of the transgene. We used Thermal Asymmetric Interlaced PCR (TAIL-PCR) (Liu and Whittier, 1995; Pillai et al., 2007)to

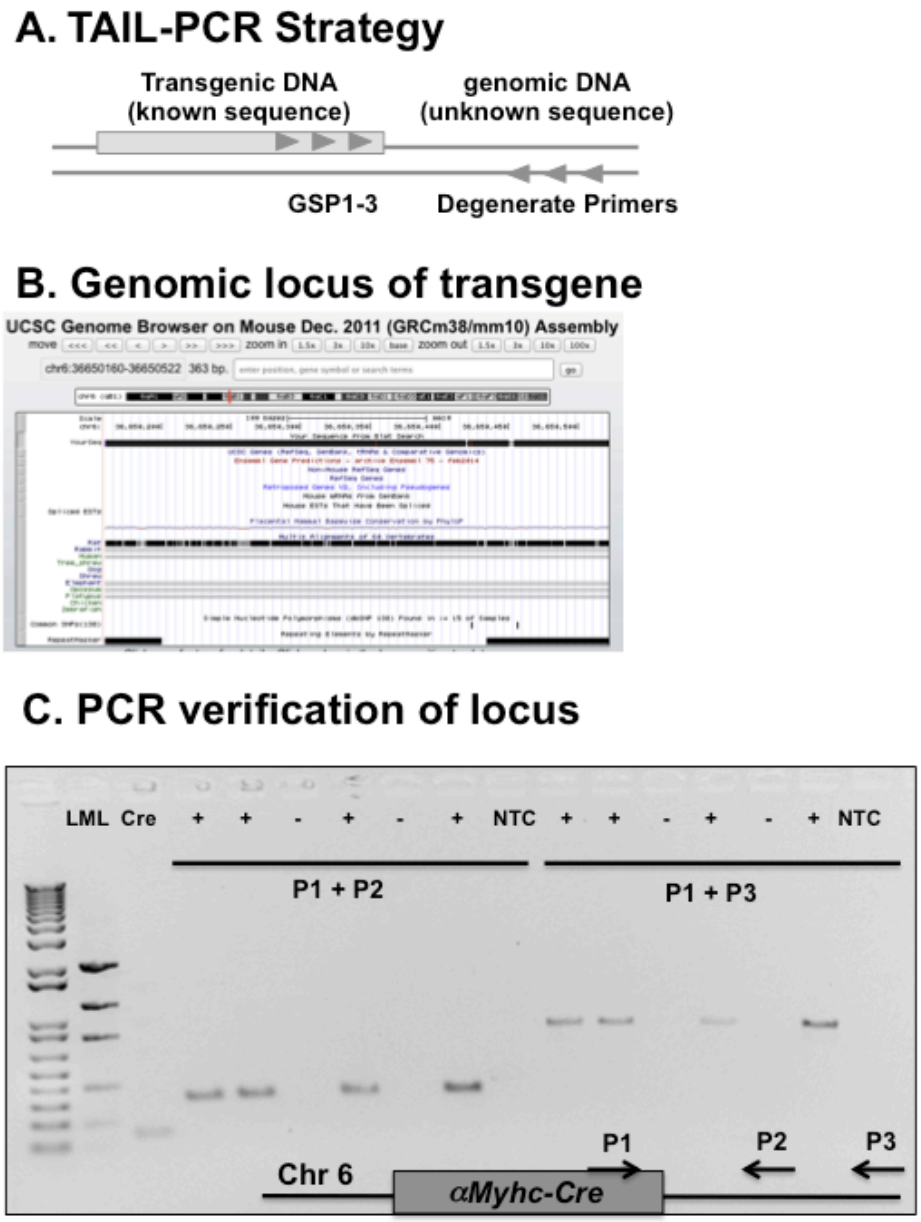

Figure 4.12: Genomic insertion site of *MyHC-Cre* transgene does not likely account for cardiotoxicity. (A) PCR-based strategy for mapping transgene insertion site utilizes sequential, forward gene-specific primers (GSP1-3) in 3' end of *MyHC-Cre* transgene and degenerate, reverse primers in flanking genomic DNA. **(B)** Screenshot of UCSC Genome Browser window displaying Chromosome 6 genomic locus of transgene to which PCR products were mapped. The region is intergenic and non-transcriptionally active based on displayed Genome Browser tracks. **(C)** Genomic locus was verified using PCR of additional animals. Products of expected length based on primer design were only amplified in *MyHC-Cre*$^{+/-}$ mice. Lane 1: 1kb+ DNA ladder (Invitrogen), Lane 2: LML (low mass ladder, Invitrogen), Lane 3: *Cre* PCR product, positive control, Lanes 4-17: *MyHC-Cre* genotype (+ or -) or no template control (NTC).

map the insertion site of the α*MyHC-Cre* transgene (Figure 4.12). By this method, 6/10 sequenced PCR products mapped to an intergenic locus on Chromosome 6 and 4/10 products mapped to the 5' end of the transgene, suggesting tandem insertion (Figure 4.12B). We then used qPCR of gDNA in conjunction with standard curve generation to determine copy number of the α*MyHC-Cre* transgene (Joshi et al., 2008). By this method, copy number was estimated to be 6 copies/genome which we believe to be tandem insertions based on our results from TAIL-PCR (Figure 4.13). Taken together, we do not believe insertion site or high copy number of the *αMyHC-Cre* transgene can account for the progressive cardiotoxicity observed in these mice.

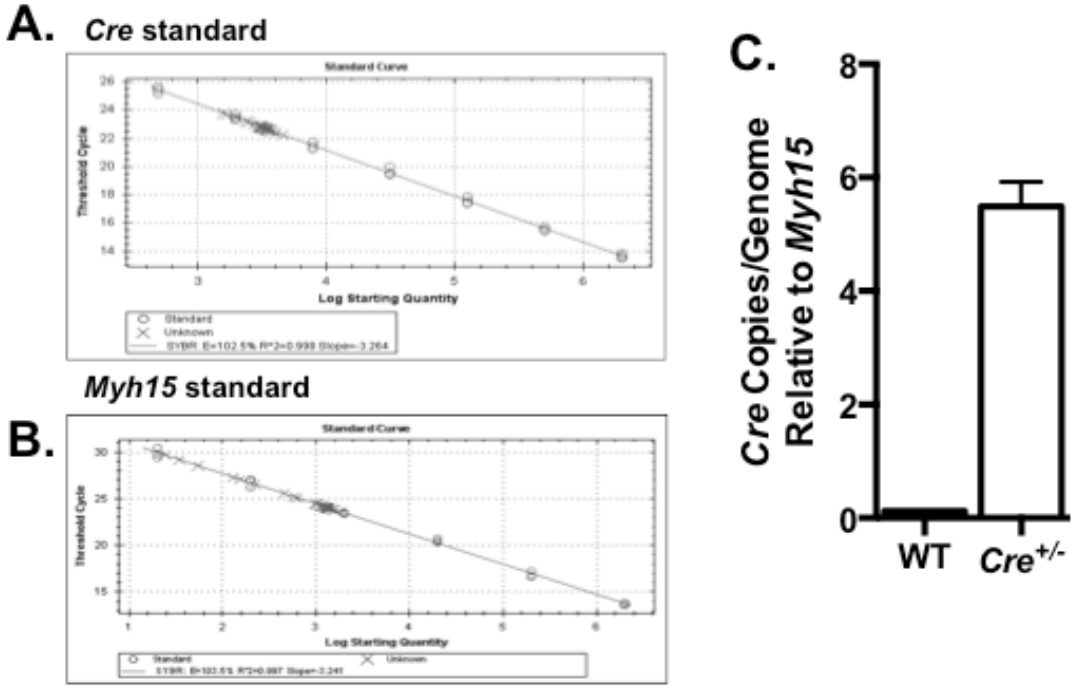

Figure 4.13: *αMyHC-Cre* transgene copy number in *αMyHC-Cre*$^{+/-}$ mice. Copy number was estimated based on standard curves generated with known copy numbers of either *Cre* **(A)** or a control gene, *Myh15* (2 copies/genome) **(B)**. **(C)** An average of 6 copies of the *αMyHC-Cre* transgene were detected per genome, when normalized to *Myh15* copy number, N=8.

4.4 Discussion

Here we present evidence that prolonged exposure to Cre recombinase in cardiac myocytes has deleterious effects on cardiac function. Although we are not the first to report cardiotoxicity associated with Cre expression in the heart, we believe this is the first characterization of such in the widely used *αMyHC-Cre* mouse line (Davis et al., 2012; Doetschman and Azhar, 2012; Molkentin and Robbins, 2009). Our findings of depressed cardiac function, an activated DNA damage response, modest but statistically significant fibrosis, and mild inflammation in α*MyHC-Cre*$^{+/-}$ myocardium are consistent with findings in inducible cardiac-specific *MerCreMer* models and other models of myocardial Cre expression (Bersell et al., 2013; Buerger et al., 2006; Lexow et al., 2013).

The early molecular changes observed in the α*MyHC-Cre*$^{+/-}$ mice precede the more robust molecular and functional changes observed at 6 months. Given the dynamic cross-regulation of the DNA damage response pathway, p38 MAPK stress response pathway, and inflammatory response, we cannot conclude that DNA damage is the primary insult (Guan et al., 1997; Molkentin et al., 1998; Zechner et al., 1997). However, given prolonged, high Cre expression, Cre's role as a DNA recombinase with promiscuous activity, and the presence of degenerate *loxP* sites in the mouse genome (Figure 4.10), we suggest this as a potential mechanism of cardiotoxicity in conjunction with an inflammatory response activated by long-term exposure to a non-endogenous DNA recombinase. Further, in a skin model of Cre toxicity, DNA damage response was shown to be dependent on Cre recombinase activity as an endonuclease-deficient mutant Cre mutant did not activate this pathway (Janbandhu et al., 2013).

Although we did not observe gross changes in gene expression at many of the

genes containing putative *loxP* sites (Figure 4.11A), this does not rule out an age or time-point dependent change in expression or genomic insults at the hundreds of intergenic sites also identified but not examined (Figure 4.10). Further, genomic changes are likely heterogeneous among cardiac myocytes so detecting changes in individual genes from a population of myocytes may be difficult. It is likely that genomic sites with high accessibility or sites that are more closely matched to the canonical *loxP* site may be more often targeted and more easily identified by changes in gene expression (as in the case of *Me3* or *Ptgfr*, Figure 4.11A). Although beyond the scope of this report, whole genome sequencing of α*MyHC-Cre* cardiac myocytes may be a preferred method for addressing Cre's effect on genomic integrity.

Genetic background, diet, and vivarium conditions may all play a part in the Cre-associated phenotype we observed. Cardiac outcomes can be heavily influenced by genetic background in particular (Qiu et al., 2009; Sanford et al., 2001; Schulkey et al., 2015; Semsarian et al., 2001). We chose to perform our studies in the C57Bl/6J background given the wide usage of this isogenic strain however it is unclear to what extent other strains are susceptible to Cre-related cardiotoxicity. Interestingly, α*MyHC-Cre* mice on a mixed genetic background appear more resistant to cardiotoxicity than those observed in this study (Bergo et al., 2003). Similar results were observed for α*MyHC-MerCreMer* mice in which animals on a mixed background (129Ola/C57Bl/6J) displayed less fibrosis than pure C57Bl/6J mice (Lexow et al., 2013).

Sexually dimorphic phenotypes were observed at both timepoints examined, both by echocardiography and morphometric analysis (Figure 4.4 and Table 4.1). Our subsequent analyses focused on female mice, given their demonstrated resistance to

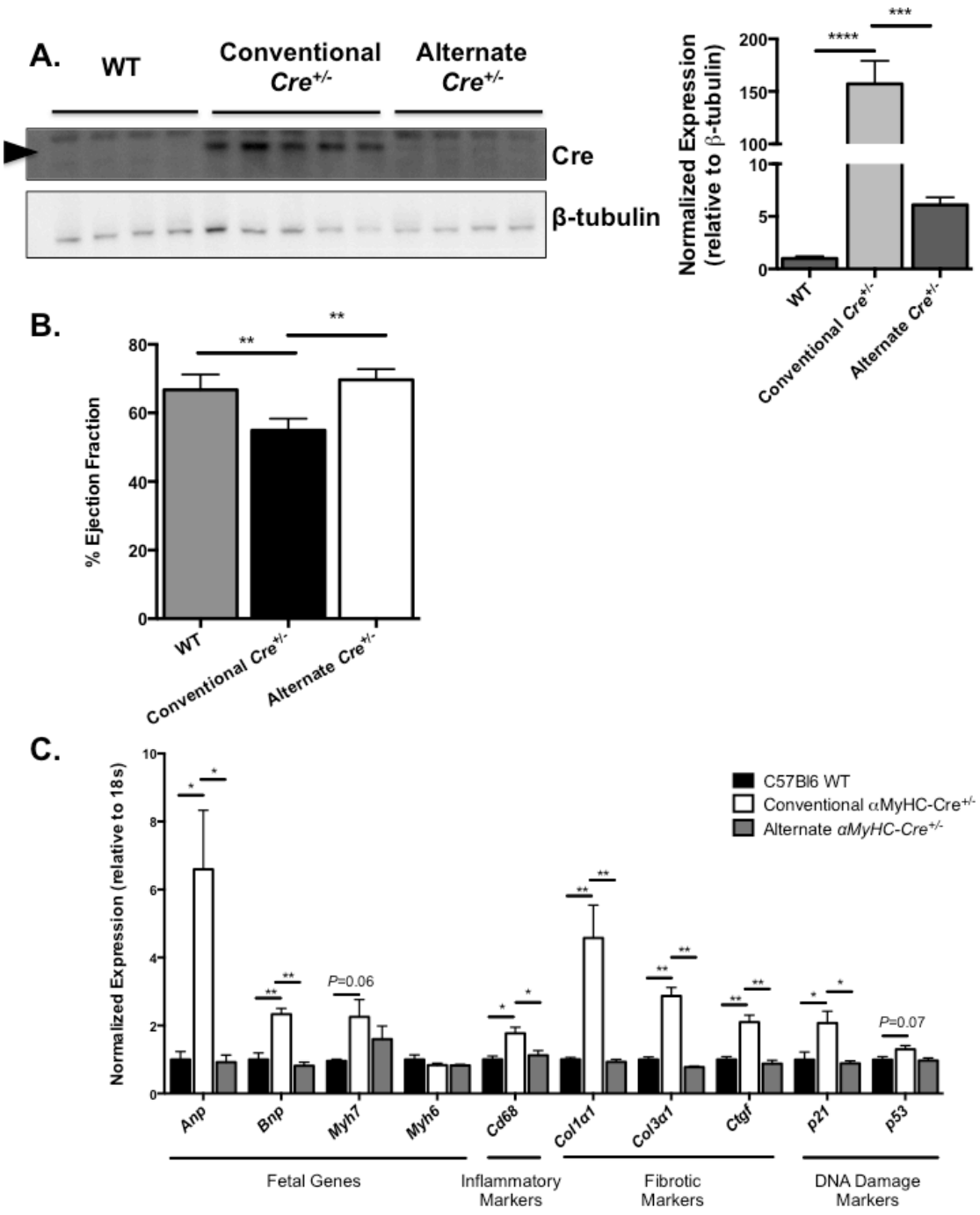

Figure 4.14: Lower levels of myocardial Cre expression do not result in cardiotoxicity. Cre expression and cardiotoxicity were assessed in male WT, "conventional" *αMyHC-Cre*$^{+/-}$ mice (Agah, Frenkel et al. 1997) and "alternate" *αMyHC-Cre*$^{+/-}$ mice (Abel, Kaulbach et al. 1999). Although both transgenes drive *Cre* expression under the *MyHC* promoter, the transgene structures differ in their 3'UTRs and promoter sequences (Agah, Frenkel et al. 1997, Abel, Kaulbach et al. 1999). Differences in transgene structure, copy number and insertion site may all contribute to expression differences in Cre.

(A) Cre protein expression (immunoblot). **(B)** Cardiac function (%EF). **(C)** Gene expression (qRT-PCR). Error bars: SEM, N=4-5/group, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ vs. age-matched and sex-matched WT controls.

cardiac insult compared to males (Cavasin et al., 2004; Douglas et al., 1998). Future studies are needed to more carefully dissect differential hypertrophic and molecular changes in α*MyHC-Cre* males, although many of the gene expression changes observed in females were also confirmed in males (Figure 4.8). Although we did not further investigate the sexually dimorphic nature of our findings, they support the need for sex-specific genetic studies.

Given the high promoter activity of α*MyHC* in cardiac myocytes, it is perhaps not surprising that the resulting, persistent expression of *Cre* eventually leads to pathological remodeling. Interestingly, in another ("alternate") model of cardiomyocyte-specific Cre expression (Abel et al., 1999) in which myocardial Cre protein expression is considerably lower than the more commonly used, "conventional" *αMyHC* mice (Agah et al., 1997), cardiotoxicity was not observed in age-matched mice (Figure 4.14). This supports our hypothesis that Cre-related toxicity may be time and dose-dependent.

Since the efficiency of Cre to induce recombination of canonical *loxP* sites is quite high (Rufer and Sauer, 2002) and cardiac myocytes rarely divide, we suggest using a temporally restricted or dose-dependent promoter to drive *Cre* expression. Others have shown that even one dose of tamoxifen is sufficient to induce recombination in an inducible *αMyHC-MerCreMer* model (Lexow et al., 2013). However, some genomic loci are resistant to recombination and require more persistent *Cre* expression. In light of these issues and the data we present, we propose that a less cardiotoxic model of cardiac

myocyte-specific genome editing is needed for the field. Until this is introduced, we strongly urge investigators to be cautious in their choice of *Cre* driver and to always include a *Cre*-only control group, even in studies of young animals (< 3 months old). Although we did not observe profound molecular changes at 3 months, it may be that *Cre* expression in cardiac myocytes sensitizes young mice to other pathological stimuli, especially those characterized by DNA damage (e.g. myocardial infarction (Itoh et al., 1995). Finally, we strongly encourage the use and separation of both sexes as molecular and phenotypic differences are common and clinically relevant.

Chapter 5

Conclusions and future directions

In this thesis work I reveal the primary cardiac myocyte Estrogen Receptor, *ERα,* and suggest its principal mechanism of signaling is through transcriptional regulation in this critically important cardiac cell type. Further, my data suggest that although *ERα* is the predominant ER in adult cardiac myocytes, its role is relatively minor in overall myocyte biology but differs between males and females. In response to cardiac stress, *ERα* has a functional requirement in the cardiac myocyte that depends upon the nature of the cardiac stressor. In pursuit of a more refined definition of *ERα* regulation of myocyte biology I discovered and characterized profound cardiotoxicity associated with cardiac myocyte-specific *Cre* expression. Although these findings better illuminate the specific function of *ERα* in cardiac myocytes, a number of questions arise from these new data.

ERα *signaling in cardiac myocytes*

ERα localization and signaling in isolated neonatal cardiac myocytes were extensively characterized in Chapter 2, but it is unclear how these findings could translate to adult cardiac myocytes. Although ERα is not highly expressed in neonatal myocytes, these cells were chosen for *in vitro* studies for several reasons. First, neonatal cardiac myocytes are structurally and functionally similar to adult myocytes (Harary and Farley, 1960; Simpson et al., 1982). In addition, the gene expression profile of neonatal cardiac myocytes can be considered ERα-negative (Figure 2.2) so neonatal cells are a clean background for overexpression of individual ERα isoforms. Finally, the duration of the studies and increased tractability of neonatal cardiac myocytes combined with the poor *ex*

vivo survival of adult myocytes made neonatal cells more amenable to the analyses undertaken.

Although adult myocytes were not as rigorously explored for ERα-mediated signaling effects, full-length EGFP-tagged ERα localization was assessed in these cells. In agreement with data from neonatal cardiac myocytes, ERα66 was found to primarily localize to female myocyte nuclei (Figure 2.11 and Figure 5.1). More careful characterization of differential ERα isoform localization, signaling, and transcriptional activity in both male and female adult cardiac myocytes is required to determine whether ERα exerts a similar mechanism of transcriptional regulation in neonatal and adult cardiac myocytes.

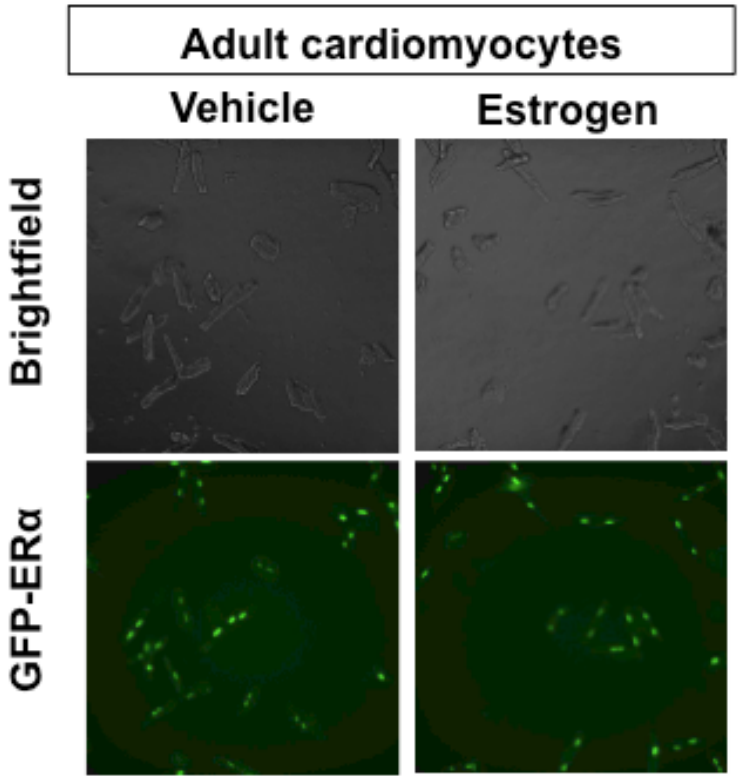

Figure 5.1: GFP-ERα localization in adult cardiac myocytes. Female adult rat ventricular myocytes were infected with adenovirus containing EGFP-ERα66. EGFP-ERα66 localizes primarily to nuclear structures in binucleate adult myocytes.

As discussed in Chapter 2, the functional relevance of alternate *ERα* spliceforms in cardiac myocytes is as of yet unknown. Although ERα46 nor ERα36 have been exclusively reported and studied in human cells, studies in human cardiac myocytes are not technically feasible so rodent myocyte studies are a viable alternative. Interestingly, in a pilot study, ERα36-specific transcript was detected in human ventricular myocardium (data not shown) suggesting a role for this ERα isoform in one or more cell types in the adult heart.

In vivo role for ERα *in cardiac myocytes*

As demonstrated in Chapter 3, the consequences of *in vivo* targeted *ERα* deletion in cardiac myocytes are apparently modest in unstressed animals. The response of these *ERα* cKO mice to different forms of cardiac stress is more curious. Although suggestive of a role for *ERα* in physiological and pathological myocardial remodeling, data presented in Chapter 4 demand that the response of *αMyHC-Cre*$^{+/-}$ mice to the same cardiac stressors be directly compared to the response of *ERα* cKO mice. The influence of *ERα* on *Cre* related cardiotoxicity is also worth additional consideration.

Regardless of the influence of myocardial *Cre* expression on the *ERα* phenotypes observed, the question of how ERα differentially mediates physiological and pathological remodeling is still valid and potentially quite meaningful. Perhaps even more relevant to hormone-regulation of cardiac myocyte biology would be an investigation of the response of *ERα* cKO females to pregnancy-induced cardiac remodeling, a sex-specific form of cardiac stress that is generally considered physiological (Chung and Leinwand, 2014).

Myocardial Cre *expression*

The data presented in Chapter 4 provides unconditional support for inclusion of a *Cre*-only genotype in all *in vivo* studies that require cardiac myocyte *Cre* expression. However, the costs of adding an additional genotype to any given experiment can be quite high. With this in mind, the findings published in Chapter 4 will at the very least provide a baseline comparison for subsequent studies should this additional, critical control not be included for financial or other logistical reasons (Pugach et al., 2015).

A key question raised in Chapter 4 is whether the putative, degenerate "lox-like" sites identified actually undergo Cre-induced recombination. Although changes in gene expression suggest targeting of the loci identified, Cre binding and/or recombination cannot be definitively proven. One or several of the gene expression changes observed in Figure 4.11 may be attributable or secondary to other changes in overall cardiac health, as suggested by a recent commentary (Bhandary and Robbins, 2015). In order to address this issue, the loci identified must be sequenced. Further biochemical analysis of *Cre* binding to the identified loci could also lend support to a mechanism of direct transcription inhibition, as shown previously (Iovino et al., 2005). The extent of heterogeneity among individual cells and endogenous, degenerate, *loxP* sites is also worth investigating although the technical issues related to high-coverage, single-cell sequencing present a significant challenge to addressing this question. Of note, it has been previously shown that certain endogenous *loxP*-like sites are competent for Cre-based insertional transgenesis using corresponding degenerate *loxP*-like sites to target recombination (Thyagarajan et al., 2000).

Finally, the kinetics of off-target Cre targeting and recombination remain somewhat puzzling. The relatively long incubation period for Cre-mediated cardiotoxicity is

somewhat recapitulated *in vitro* as isolated, neonatal cardiac myocytes are relatively resistant to Cre-induced toxicity (Figure 5.2). It is difficult to model the *in vivo* dynamics of Cre-induced DNA damage and toxicity *in vitro* given the short period of time in which neonatal or adult cardiac myocytes can be cultured. The data in Chapter 4 and the known kinetics of Cre binding and recombination at certain degenerate sites together support a model in which slow accumulation of genetic lesions in terminally differentiated cardiac myocytes gradually result in DNA damage response, myocyte loss, and fibrotic deposition (Thyagarajan et al., 2000). The dose-dependent effect of Cre expression on degree of cardiotoxicity further supports this model (Figure 4.5 and Figure 4.14).

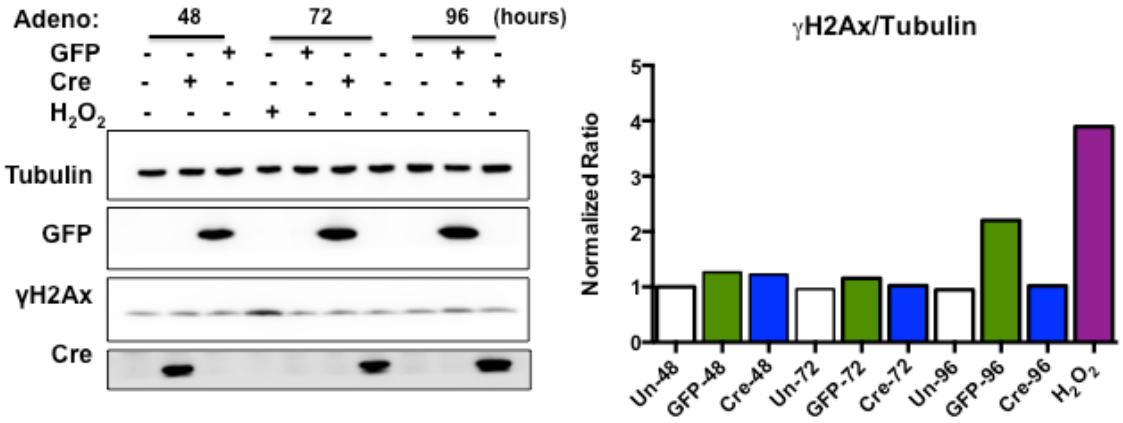

Figure 5.2: Neonatal ventricular cardiac myocytes are resistant to Cre-induced toxicity and DNA damage. Neonatal rat ventricular myocytes were infected with adenovirus containing *Cre* or control adenovirus containing *eGFP*. Cells were harvested after 48, 72, or 96 hours of adenoviral infection and probed for evidence of DNA damage (γH2Ax). Positive control: 10 minutes treatment with H_2O_2.

Appendix I. Primer sequences.

Primer/Gene Name	Sequence
18s 3'	ctttcgctctggtcgtctt
18s 5'	gccgctagaggtgaaattcttg
Acta1 3'	agcctcgtcgtactcctgcttgg
Acta1 5'	cgacatcaggaaggacctgtatgcc
Anp 3'	gaagctgttgcagcctagtc
Anp 5'	ccaggccatattggagcaaa
Atp6v0d 5'	caatgccattctggtggac
Atp6v0d1 3'	tttcggattatctcgatgttca
Bmpr2 3'	gtatcgaccccgtccaatc
Bmpr2 5'	gagccctcccttgacctg
Bnp 3'	ttggtccttcaagagctgtc
Bnp 5'	aaggtgctgtcccagatg
Cbfa2t2 3'	gccgctcccagtccttat
Cbfa2t2 5'	tgagcaagaagagtccacagag
CD68 3'	cggatttgaatttgggcttg
CD68 5'	ttgggaactacacacgtgggc
Chrm2 3'	gcagggttgatggtgctatt
Chrm2 5'	tggctatcctgttggctttc
Cobll1 3'	gggagagctcagttccaaca
Cobll1 5'	ggaactctgcaatggctca
Col1a1 3'	aacgggtccccttgggcctt
Col1a1 5'	aatggcacggctgtgtgcga
Col3a1 3'	tgacatggttctggcttcca
Col3a1 5'	tggcacagcagtccaacgta
Cre 3'	gctaaccagcgttttcgttc
Cre 5'	gcatttctggggattgctta
Dennda1a 3'	ggacagccactgatggtaca
Dennda1a 5'	ggtgaaggtttcagcgatgt
Dystrophin 3'	aacaagtggtttgggtctcg
Dystrophin 5'	caagcttactcctccgctctaa
Ears2 3'	ccaggtctttctaaaagcctaaaag
Ears2 5'	cggaaaacgtggatgtgatt
Elk1 3	gggtgcaattggactcaga
Elk1 5'	gctccccacacataccttga
ER-alpha 3'	agccagcaacatgtcaaaga
ER-alpha 5'	gggaagctcctgtttgctc
ER-alpha geno 3'	tgcagcagaaggtatttgcctgtta
ER-alpha geno 5'	ttgcccgataacaataacat
Fbxo40 3'	tccctgcttctggcttctta
Fbxo40 5'	tgcttgatcaacggcaaag
Fdx1 3'	caattaataaagatgtcagggacca
Fdx1 5'	tgacctggcttttggactaac
Gata4 3'	Catggccccacaattgac
Gata4 5'	Ggaagacaccccaatctcg
Hagh 3'	gcggtcatcacctccataaa
Hagh 5'	cacagtgctcaccactcacc
IL-1a 3'	agcgctcaaggagaagacc
IL-1a 5'	ccagaagaaaatgaggtcgg

IL-1β 3'	tgtgaaatgccacctttga
IL-1β 5'	ggtcaaaggtttggaagcggtag
Inadl 3'	aggccaaacgtttcttcca
Inadl 5'	tgcatgctaacctctcagga
Mcip1.4 3'	tggaaggtggtgtccttgt
Mcip1.4 5'	agctccctgattgcttgtgt
Me3 3'	tgaggcaatttattccgaac
Me3 5'	cgtggggaggagtatgatga
Mef2a 3'	ggagttagcacatatgaagtatcagg
Mef2a 5'	acgaactcggatatcgttgag
MHCre Geno 3'	ctcatcactcgttgcatcgac
MHCre Geno 5'	atgacagacagatccctcctatctcc
Myh6 3'	ctccttgtcatcaggcac
Myh6 5'	acattcttcaggattctctg
Myh7 3'	cttctcagacttccgcag
Myh7 5'	ttccttacttgctaccctc
Nfatc1 3'	tccgatgtgctgaattaggag
Nfatc1 5'	ttggaccagttgtacttggatg
p21 3'	ggacatcaccaggattggac
p21 5'	tccacagcgatatccagaca
p53 3'	agggagctcgaggctgata
p53 5'	acgcttctccgaagactgg
Pde7b 3'	gctgctgttcgatatcctgtg
Pde7b 5'	caattggcatgcttcgaga
Pgr 3'	tggtcatcgatgtgtaagttcc
Pgr 5'	ggcagctgctttcagtagtca
Pofut1 3'	acgaggagacacagtttccaa
Pofut1 5'	agcagctcttcaaagacaaggt
Ptgfr 3'	tgcaatgttggccattgtta
Ptgfr 5'	ctggccataatgtgcgtct
Rasal2 3'	tgacatcaccaagtcagtcaga
Rasal2 5'	tggcttgtgccttagtacacat
Sec22b 3'	caccccctgctcaataatgt
Sec22b5 5'	agtcccctacccgatgtacc
Stard13 3'	agaaaccaggtcatgcaagg
Stard13 5'	cggccatcatggagaagta
Stat5b 3'	ctggctgccgtgaacaat
Stat5b 5'	cgagctggtctttcaagtca
Stip1 3'	tataagccaagcgctcctgt
Stip1 5'	accccagatgtgctcaagaa
Tfpi 3'	ccactgtctgctggttgaag
Tfpi 5'	taacatcgtggttccccagt
Tnfa 3'	ccaccacgctcttctgtctac
Tnfa 5'	agggtctgggccatagaact
Tpk1 3'	tgtctacatggagcctgtgc
Tpk1 5'	catcactcctgtgccgatta
Zeb1 3'	tatcacaatacgggcaggtg
Zeb1 5'	gccagcagtcatgatgaaaa

Appendix II. Published Usage of α*MyHC* mice.

Citation	Control Mice Specified	Age of Mice
Gutstein DE, Morley GE, Tamaddon H, Vaidya D, Schneider MD, Chen J, et al. Conduction slowing and sudden arrhythmic death in mice with cardiac-restricted inactivation of connexin43. Circulation Research 2001;88:333–9.	fl/fl or not specified	Up to 60 days
Gaussin V, Van de Putte T, Mishina Y, Hanks MC, Zwijsen A, Huylebroeck D, et al. Endocardial cushion and myocardial defects after cardiac myocyte-specific conditional deletion of the bone morphogenetic protein receptor ALK3. PNAS 2002;99:2878–83.	$Cre^{Negative}$	Embryonic
Bjorkegren J, Véniant M, Kim SK, Withycombe SK, Wood PA, Hellerstein MK, et al. Lipoprotein secretion and triglyceride stores in the heart. J Biol Chem 2001;276:38511–7.	αMyHC-Cre and fl/fl	Adult
Gutstein DE, Morley GE, Fishman GI. Conditional gene targeting of connexin43: exploring the consequences of gap junction remodeling in the heart. Cell Commun Adhes 2001;8:345–8.	Not specified	Not specified
Shai S-Y, Harpf AE, Babbitt CJ, Jordan MC, Fishbein MC, Chen J, et al. Cardiac myocyte-specific excision of the beta1 integrin gene results in myocardial fibrosis and cardiac failure. Circulation Research 2002;90:458–64.	WT or fl/fl	to 6 months
Holtwick R, van Eickels M, Skryabin BV, Baba HA, Bubikat A, Begrow F, et al. Pressure-independent cardiac hypertrophy in mice with cardiomyocyte-restricted inactivation of the atrial natriuretic peptide receptor guanylyl cyclase-A. J Clin Invest 2003;111:1399–407.	fl/fl	4-7 months
Jacoby JJ, Kalinowski A, Liu M-G, Zhang SS-M, Gao Q, Chai G-X, et al. Cardiomyocyte-restricted knockout of STAT3 results in higher sensitivity to inflammation, cardiac fibrosis, and heart failure with advanced age. PNAS 2003;100:12929–34.	WT, fl/+, and fl/+;αMyHC-Cre	9 months
Gotthardt M, Hammer RE, Hübner N, Monti J, Witt CC, McNabb M, et al. Conditional expression of mutant M-line titins results in cardiomyopathy with altered sarcomere structure. J Biol Chem 2003;278:6059–65.	WT	4 weeks
Kedzierski RM, Grayburn PA, Kisanuki YY, Williams CS, Hammer RE, Richardson JA, et al. Cardiomyocyte-specific endothelin A receptor knockout mice have normal cardiac function and an unaltered hypertrophic response to angiotensin II and isoproterenol. Mol Cell Biol 2003;23:8226–32.	$Cre^{Negative}$	10 weeks
Bergo MO, Lieu HD, Gavino BJ, Ambroziak P, Otto JC, Casey PJ, et al. On the physiological importance of endoproteolysis of CAAX proteins: heart-specific RCE1 knockout mice develop a lethal cardiomyopathy. J Biol Chem 2004;279:4729–36.	αMyHC-Cre and fl/fl	to 10 months
Cheng L, Ding G, Qin Q, Huang Y, Lewis W, He N, et al. Cardiomyocyte-restricted peroxisome proliferator-activated receptor-delta deletion perturbs myocardial fatty acid oxidation and leads to cardiomyopathy. Nat Med 2004;10:1245–50.	αMyHC-Cre	Up to 1 year
Yamaguchi O, Watanabe T, Nishida K, Kashiwase K, Higuchi Y, Takeda T, et al. Cardiac-specific disruption of the c-raf-1 gene induces cardiac dysfunction and apoptosis. J Clin Invest 2004;114:937–43.	fl/fl	15 weeks
ishida K, Yamaguchi O, Hirotani S, Hikoso S, Higuchi Y, Watanabe T, et al. p38alpha mitogen-activated protein kinase plays a critical	fl/fl	8-11 weeks

role in cardiomyocyte survival but not in cardiac hypertrophic growth in response to pressure overload. Mol Cell Biol 2004;24:10611–20.		
Augustus A, Yagyu H, Haemmerle G, Bensadoun A, Vikramadithyan RK, Park S-Y, et al. Cardiac-specific knock-out of lipoprotein lipase alters plasma lipoprotein triglyceride metabolism and cardiac gene expression. J Biol Chem 2004;279:25050–7.	WT	2 months
Shohet RV, Kisanuki YY, Zhao X-S, Siddiquee Z, Franco F, Yanagisawa M. Mice with cardiomyocyte-specific disruption of the endothelin-1 gene are resistant to hyperthyroid cardiac hypertrophy. PNAS 2004;101:2088–93.	fl/fl	15-19 weeks
Schaeffer PJ, Wende AR, Magee CJ, Neilson JR, Leone TC, Chen F, et al. Calcineurin and calcium/calmodulin-dependent protein kinase activate distinct metabolic gene regulatory programs in cardiac muscle. J Biol Chem 2004;279:39593–603.	fl/fl	Not specified
Skryabin BV, Holtwick R, Fabritz L, Kruse MN, Veltrup I, Stypmann J, et al. Hypervolemic hypertension in mice with systemic inactivation of the (floxed) guanylyl cyclase-A gene by alphaMHC-Cre-mediated recombination. Genesis 2004;39:288–98.	fl/fl	4-12 months
McFadden DG, Barbosa AC, Richardson JA, Schneider MD, Srivastava D, Olson EN. The Hand1 and Hand2 transcription factors regulate expansion of the embryonic cardiac ventricles in a gene dosage-dependent manner. Development 2005;132:189–201.	WT	Embryonic, perinatal
Freund C, Schmidt-Ullrich R, Baurand A, Dunger S, Schneider W, Loser P, et al. Requirement of nuclear factor-kappaB in angiotensin II- and isoproterenol-induced cardiac hypertrophy in vivo. Circulation 2005;111:2319–25.	fl/fl	12-18 weeks
MacLellan WR, Garcia A, Oh H, Frenkel P, Jordan MC, Roos KP, et al. Overlapping roles of pocket proteins in the myocardium are unmasked by germ line deletion of p130 plus heart-specific deletion of Rb. Mol Cell Biol 2005;25:2486–97.	αMyHC-Cre (homozygous)	8-16 weeks
Vong LH, Ragusa MJ, Schwarz JJ. Generation of conditional Mef2cloxP/loxP mice for temporal- and tissue-specific analyses. Genesis 2005;43:43–8.	Not specified or αMyHC-Cre;fl/+	to 40 weeks
van Oort RJ, van Rooij E, Bourajjaj M, Schimmel J, Jansen MA, van der Nagel R, et al. MEF2 activates a genetic program promoting chamber dilation and contractile dysfunction in calcineurin-induced heart failure. Circulation 2006;114:298–308.	αMyHC-Cre	8-12 weeks
Eckardt D, Kirchhoff S, Kim J-S, Degen J, Theis M, Ott T, et al. Cardiomyocyte-restricted deletion of connexin43 during mouse development. Journal of Molecular and Cellular Cardiology 2006;41:963–71.	fl/+ and fl/fl	to 12 days
Garcia-Gras E, Lombardi R, Giocondo MJ, Willerson JT, Schneider MD, Khoury DS, et al. Suppression of canonical Wnt/beta-catenin signaling by nuclear plakoglobin recapitulates phenotype of arrhythmogenic right ventricular cardiomyopathy. J Clin Invest 2006;116:2012–21.	fl/fl, fl/+, αMyHC-Cre, and WT in supplement	Embryonic
Chen J-F, Murchison EP, Tang R, Callis TE, Tatsuguchi M, Deng Z, et al. Targeted deletion of Dicer in the heart leads to dilated cardiomyopathy and heart failure. PNAS 2008;105:2111–6.	WT	Embryonic, perinatal
Montgomery RL, Davis CA, Potthoff MJ, Haberland M, Fielitz J, Qi X, et al. Histone deacetylases 1 and 2 redundantly regulate cardiac morphogenesis, growth, and contractility. Genes Dev 2007;21:1790–802.	WT	8 weeks + 2 weeks of aortic banding or Isoproteranol

Hoogaars WMH, Engel A, Brons JF, Verkerk AO, de Lange FJ, Wong LYE, et al. Tbx3 controls the sinoatrial node gene program and imposes pacemaker function on the atria. Genes Dev 2007;21:1098–112.	αMyHC-Cre	Embryonic
Fielitz J, Kim M-S, Shelton JM, Qi X, Hill JA, Richardson JA, et al. Requirement of protein kinase D1 for pathological cardiac remodeling. PNAS 2008;105:3059–63.	WT	8-10 weeks + 3 weeks TAC
Ding G, Fu M, Qin Q, Lewis W, Kim HW, Fukai T, et al. Cardiac peroxisome proliferator-activated receptor gamma is essential in protecting cardiomyocytes from oxidative damage. Cardiovasc Res 2007;76:269–79.	αMyHC-Cre	3-4 months
Nakaoka Y, Nishida K, Narimatsu M, Kamiya A, Minami T, Sawa H, et al. Gab family proteins are essential for postnatal maintenance of cardiac function via neuregulin-1/ErbB signaling. J Clin Invest 2007;117:1771–81.	fl/fl; single cKOs	12-72 weeks
Qu J, Zhou J, Yi XP, Dong B, Zheng H, Miller LM, et al. Cardiac-specific haploinsufficiency of beta-catenin attenuates cardiac hypertrophy but enhances fetal gene expression in response to aortic constriction. Journal of Molecular and Cellular Cardiology 2007;43:319–26.	Pilot showed no differences between αMy HC-Cre and controls used (fl/+)	4 months + TAC
Xiong S, Van Pelt CS, Elizondo-Fraire AC, Fernandez-Garcia B, Lozano G. Loss of Mdm4 results in p53-dependent dilated cardiomyopathy. Circulation 2007;115:2925–30.	αMyHC-Cre; fl/+	to 8 months
Malester B, Tong X, Ghiu I, Kontogeorgis A, Gutstein DE, Xu J, et al. Transgenic expression of a dominant negative K(ATP) channel subunit in the mouse endothelium: effects on coronary flow and endothelin-1 secretion. Faseb J 2007;21:2162–72.	$Cre^{Negative}$	Not specified
Chang GY, Cao F, Krishnan M, Huang M, Li Z, Xie X, et al. Positron emission tomography imaging of conditional gene activation in the heart. Journal of Molecular and Cellular Cardiology 2007;43:18–26.	$Cre^{Negative}$	20-30g mice
Engleka KA, Wu M, Zhang M, Antonucci NB, Epstein JA. Menin is required in cranial neural crest for palatogenesis and perinatal viability. Dev Biol 2007;311:524–37.	Not shown	>P0
Matus M, Lewin G, Stümpel F, Buchwalow IB, Schneider MD, Schütz G, et al. Cardiomyocyte-specific inactivation of transcription factor CREB in mice. Faseb J 2007;21:1884–92.	fl/fl	16-24 weeks
Montgomery RL, Potthoff MJ, Haberland M, Qi X, Matsuzaki S, Humphries KM, et al. Maintenance of cardiac energy metabolism by histone deacetylase 3 in mice. J Clin Invest 2008;118:3588–97.	fl/fl	3-4 months
Lai L, Leone TC, Zechner C, Schaeffer PJ, Kelly SM, Flanagan DP, et al. Transcriptional coactivators PGC-1alpha and PGC-lbeta control overlapping programs required for perinatal maturation of the heart. Genes Dev 2008;22:1948–61.	WT, fl/fl	P0-P28
Raake PW, Vinge LE, Gao E, Boucher M, Rengo G, Chen X, et al. G protein-coupled receptor kinase 2 ablation in cardiac myocytes before or after myocardial infarction prevents heart failure. Circulation Research 2008;103:413–22.	fl/fl	8 weeks + 30 days post myocardial infarction
Dobrowolski R, Sasse P, Schrickel JW, Watkins M, Kim J-S, Rackauskas M, et al. The conditional connexin43G138R mouse mutant represents a new model of hereditary oculodentodigital dysplasia in humans. Hum Mol Genet 2008;17:539–54.	WT	6.5 months survival
Lisewski U, Shi Y, Wrackmeyer U, Fischer R, Chen C, Schirdewan A, et al. The tight junction protein CAR regulates cardiac conduction	$Cre^{Negative}$	Embryonic-P1

and cell-cell communication. J Exp Med 2008;205:2369–79.		
Lim B-K, Xiong D, Dorner A, Youn T-J, Yung A, Liu TI, et al. Coxsackievirus and adenovirus receptor (CAR) mediates atrioventricular-node function and connexin 45 localization in the murine heart. J Clin Invest 2008;118:2758–70.	WT	to 33 weeks
Kontaridis MI, Yang W, Bence KK, Cullen D, Wang B, Bodyak N, et al. Deletion of Ptpn11 (Shp2) in cardiomyocytes causes dilated cardiomyopathy via effects on the extracellular signal-regulated kinase/mitogen-activated protein kinase and RhoA signaling pathways. Circulation 2008;117:1423–35.	fl/fl	6 weeks
Levkau B, Schäfers M, Wohlschlaeger J, Wnuck Lipinski von K, Keul P, Hermann S, et al. Survivin determines cardiac function by controlling total cardiomyocyte number. Circulation 2008;117:1583–93.	$Cre^{Negative}$	20-30 weeks
Tessari A, Pietrobon M, Notte A, Cifelli G, Gage PJ, Schneider MD, et al. Myocardial Pitx2 differentially regulates the left atrial identity and ventricular asymmetric remodeling programs. Circulation Research 2008;102:813–22.	fl/fl and fl/+	Perinatal
Qian J-Y, Harding P, Liu Y, Shesely E, Yang X-P, LaPointe MC. Reduced cardiac remodeling and function in cardiac-specific EP4 receptor knockout mice with myocardial infarction. Hypertension 2008;51:560–6.	fl/fl	14 weeks
Liem DA, Zhao P, Angelis E, Chan SS, Zhang J, Wang G, et al. Cyclin-dependent kinase 2 signaling regulates myocardial ischemia/reperfusion injury. Journal of Molecular and Cellular Cardiology 2008;45:610–6.	αMyHC-Cre	Not specified
Lombardi R, Dong J, Rodriguez G, Bell A, Leung TK, Schwartz RJ, et al. Genetic fate mapping identifies second heart field progenitor cells as a source of adipocytes in arrhythmogenic right ventricular cardiomyopathy. Circulation Research 2009;104:1076–84.	WT	3-6 months
Huang J, Min Lu M, Cheng L, Yuan L-J, Zhu X, Stout AL, et al. Myocardin is required for cardiomyocyte survival and maintenance of heart function. PNAS 2009;106:18734–9.	fl/fl	to 1 year
Kratsios P, Catela C, Salimova E, Huth M, Berno V, Rosenthal N, et al. Distinct roles for cell-autonomous Notch signaling in cardiomyocytes of the embryonic and adult heart. Circulation Research 2010;106:559–72.	fl/fl	Perinatal
Xu J, Ismat FA, Wang T, Lu MM, Antonucci N, Epstein JA. Cardiomyocyte-specific loss of neurofibromin promotes cardiac hypertrophy and dysfunction. Circulation Research 2009;105:304–11.	fl/fl or fl/+	32 weeks
Schaeffer PJ, Desantiago J, Yang J, Flagg TP, Kovacs A, Weinheimer CJ, et al. Impaired contractile function and calcium handling in hearts of cardiac-specific calcineurin b1-deficient mice. Am J Physiol Heart Circ Physiol 2009;297:H1263–73.	fl/fl; originally screened αMyHC-Cre mice	270 days
Li Y, Cheng L, Qin Q, Liu J, Lo W-K, Brako LA, et al. High-fat feeding in cardiomyocyte-restricted PPARdelta knockout mice leads to cardiac overexpression of lipid metabolic genes but fails to rescue cardiac phenotypes. Journal of Molecular and Cellular Cardiology 2009;47:536–43.	αMyHC-Cre	28 weeks
Azzouzi el H, van Oort RJ, van der Nagel R, Sluiter W, Bergmann MW, De Windt LJ. MEF2 transcriptional activity maintains mitochondrial adaptation in cardiac pressure overload. Eur J Heart Fail 2010;12:4–12.	αMyHC-Cre	10-15 weeks

Kuroda J, Ago T, Matsushima S, Zhai P, Schneider MD, Sadoshima J. NADPH oxidase 4 (Nox4) is a major source of oxidative stress in the failing heart. PNAS 2010;107:15565–70.	WT	"Young"
Takeda N, Manabe I, Uchino Y, Eguchi K, Matsumoto S, Nishimura S, et al. Cardiac fibroblasts are essential for the adaptive response of the murine heart to pressure overload. J Clin Invest 2010;120:254–65.	fl/fl	8-10 weeks
Kim B-E, Turski ML, Nose Y, Casad M, Rockman HA, Thiele DJ. Cardiac copper deficiency activates a systemic signaling mechanism that communicates with the copper acquisition and storage organs. Cell Metab 2010;11:353–63.	fl/fl	1 month
Moslehi J, Minamishima YA, Shi J, Neuberg D, Charytan DM, Padera RF, et al. Loss of hypoxia-inducible factor prolyl hydroxylase activity in cardiomyocytes phenocopies ischemic cardiomyopathy. Circulation 2010;122:1004–16.	fl/fl for <5 weeks, αMyHC-Cre for survival	5-18 weeks
Kanazawa H, Ieda M, Kimura K, Arai T, Kawaguchi-Manabe H, Matsuhashi T, et al. Heart failure causes cholinergic transdifferentiation of cardiac sympathetic nerves via gp130-signaling cytokines in rodents. J Clin Invest 2010;120:408–21.	αMyHC-Cre and fl/fl	4 weeks
Liao X, Haldar SM, Lu Y, Jeyaraj D, Paruchuri K, Nahori M, et al. Krüppel-like factor 4 regulates pressure-induced cardiac hypertrophy. Journal of Molecular and Cellular Cardiology 2010;49:334–8.	αMyHC-Cre and fl/fl	2-4 months
Kratsios P, Huth M, Temmerman L, Salimova E, Banchaabouchi Al M, Sgoifo A, et al. Antioxidant amelioration of dilated cardiomyopathy caused by conditional deletion of NEMO/IKKgamma in cardiomyocytes. Circulation Research 2010;106:133–44.	αMyHC-Cre (supplemental) and fl/+	8-10 months
Maillet M, Davis J, Auger-Messier M, York A, Osinska H, Piquereau J, et al. Heart-specific deletion of CnB1 reveals multiple mechanisms whereby calcineurin regulates cardiac growth and function. J Biol Chem 2010;285:6716–24.	αMyHC-Cre	7 months
Qiu Z, Cang Y, Goff SP. c-Abl tyrosine kinase regulates cardiac growth and development. PNAS 2010;107:1136–41.	fl/fl	Embryonic
Frank M, Eiberger B, Janssen-Bienhold U, de Sevilla Müller LP, Tjarks A, Kim J-S, et al. Neuronal connexin-36 can functionally replace connexin-45 in mouse retina but not in the developing heart. J Cell Sci 2010;123:3605–15.	fl/fl	Embryonic
Papanicolaou KN, Streicher JM, Ishikawa T-O, Herschman H, Wang Y, Walsh K. Preserved heart function and maintained response to cardiac stresses in a genetic model of cardiomyocyte-targeted deficiency of cyclooxygenase-2. Journal of Molecular and Cellular Cardiology 2010;49:196–209.	fl/fl	10-16 weeks
Wendler CC, Poulsen RR, Ghatpande S, Greene RW, Rivkees SA. Identification of the heart as the critical site of adenosine mediated embryo protection. BMC Dev Biol 2010;10:57.	fl/fl	Embryonic
Papanicolaou KN, Khairallah RJ, Ngoh GA, Chikando A, Luptak I, O'Shea KM, et al. Mitofusin-2 maintains mitochondrial structure and contributes to stress-induced permeability transition in cardiac myocytes. Mol Cell Biol 2011;31:1309–28.	fl/fl and WT; αMyHC-Cre for some experiments	10 weeks
Li Y, Hiroi Y, Ngoy S, Okamoto R, Noma K, Wang C-Y, et al. Notch1 in bone marrow-derived cells mediates cardiac repair after myocardial infarction. Circulation 2011;123:866–76.	αMyHC-Cre and fl/fl	3-4 months
Li D, Liu Y, Maruyama M, Zhu W, Chen H, Zhang W, et al. Restrictive loss of plakoglobin in cardiomyocytes leads to arrhythmogenic cardiomyopathy. Hum Mol Genet 2011;20:4582–96.	fl/fl and fl/+;αMHC-Cre	1-5 months

Zingman LV, Zhu Z, Sierra A, Stepniak E, Burnett CM-L, Maksymov G, et al. Exercise-induced expression of cardiac ATP-sensitive potassium channels promotes action potential shortening and energy conservation. Journal of Molecular and Cellular Cardiology 2011;51:72–81.	$Cre^{Negative}$	8-12 weeks
Shukla PC, Singh KK, Quan A, Al-Omran M, Teoh H, Lovren F, et al. BRCA1 is an essential regulator of heart function and survival following myocardial infarction. Nat Commun 2011;2:593.	αMyHC-Cre and fl/fl	10-12 weeks
Shimano M, Ouchi N, Nakamura K, van Wijk B, Ohashi K, Asaumi Y, et al. Cardiac myocyte follistatin-like 1 functions to attenuate hypertrophy following pressure overload. PNAS 2011;108:E899–906.	αMyHC-Cre	8 weeks
Olaopa M, Zhou H-M, Snider P, Wang J, Schwartz RJ, Moon AM, et al. Pax3 is essential for normal cardiac neural crest morphogenesis but is not required during migration nor outflow tract septation. Dev Biol 2011;356:308–22.	WT	Embryonic
Piven OO, Kostetskii IE, Macewicz LL, Kolomiets YM, Radice GL, Lukash LL. Requirement for N-cadherin-catenin complex in heart development. Exp Biol Med (Maywood) 2011;236:816–22.	mix of non cKO genotypes	Embryonic, perinatal
Razani B, Zhang H, Schulze PC, Schilling JD, Verbsky J, Lodhi IJ, et al. Fatty acid synthase modulates homeostatic responses to myocardial stress. J Biol Chem 2011;286:30949–61.	αMyHC-Cre and fl/fl	3-4 months
Singh KK, Shukla PC, Quan A, Desjardins J-F, Lovren F, Pan Y, et al. BRCA2 protein deficiency exaggerates doxorubicin-induced cardiomyocyte apoptosis and cardiac failure. J Biol Chem 2012;287:6604–14.	pooled: fl/fl, αMyHC-Cre, and WT	10-12 weeks
Zhu Z, Burnett CM-L, Maksymov G, Stepniak E, Sierra A, Subbotina E, et al. Reduction in number of sarcolemmal KATP channels slows cardiac action potential duration shortening under hypoxia. Biochem Biophys Res Commun 2011;415:637–41.	WT	8-12 weeks
Shimano M, Ouchi N, Nakamura K, Oshima Y, Higuchi A, Pimentel DR, et al. Cardiac myocyte-specific ablation of follistatin-like 3 attenuates stress-induced myocardial hypertrophy. J Biol Chem 2011;286:9840–8.	αMyHC-Cre	10 weeks
Liu J, Wang P, He L, Li Y, Luo J, Cheng L, et al. Conditional PPARγ knockout from cardiomyocytes of adult mice impairs myocardial fatty acid utilization and cardiac function. PPAR Research n.d.;2011.	αMyHC-Cre	10 weeks
Bao M, Kanter EM, Huang RY-C, Maxeiner S, Frank M, Zhang Y, et al. Residual Cx45 and its relationship to Cx43 in murine ventricular myocardium. Channels (Austin) 2011;5:489–99.	WT, fl/fl, fl/+;αMyHC-Cre	Adult, not specified
Patten IS, Rana S, Shahul S, Rowe GC, Jang C, Liu L, et al. Cardiac angiogenic imbalance leads to peripartum cardiomyopathy. Nature 2012;485:333–8.	αMyHC-Cre for most experiments	Peripartum
Dillon CP, Oberst A, Weinlich R, Janke LJ, Kang T-B, Ben-Moshe T, et al. Survival function of the FADD-CASPASE-8-cFLIP(L) complex. Cell Rep 2012;1:401–7.	fl/fl or fl/+; $Cre^{Negative}$	10 weeks
Papanicolaou KN, Ngoh GA, Dabkowski ER, O'Connell KA, Ribeiro RF, Stanley WC, et al. Cardiomyocyte deletion of mitofusin-1 leads to mitochondrial fragmentation and improves tolerance to ROS-induced mitochondrial dysfunction and cell death. Am J Physiol Heart Circ Physiol 2012;302:H167–79.	Mix of αMyHC-Cre and fl/fl	3-4 mos
Gomes J, Finlay M, Ahmed AK, Ciaccio EJ, Asimaki A, Saffitz JE, et al. Electrophysiological abnormalities precede overt structural changes in arrhythmogenic right ventricular cardiomyopathy due to mutations in desmoplakin-A combined murine and human study. Eur	fl/+; $Cre^{Negative}$	2 and 6 months

Heart J 2012;33:1942–53.		
Del Re DP, Yang Y, Nakano N, Cho J, Zhai P, Yamamoto T, et al. Yes-associated protein isoform 1 (Yap1) promotes cardiomyocyte survival and growth to protect against myocardial ischemic injury. J Biol Chem 2013;288:3977–88.	fl/+; αMyHC-Cre and fl/fl	8 week old males
Oba T, Yasukawa H, Hoshijima M, Sasaki K-I, Futamata N, Fukui D, et al. Cardiac-specific deletion of SOCS-3 prevents development of left ventricular remodeling after acute myocardial infarction. J Am Coll Cardiol 2012;59:838–52.	Not specified	8-16 weeks
Kurosaka S, Leu NA, Pavlov I, Han X, Ribeiro PAB, Xu T, et al. Arginylation regulates myofibrils to maintain heart function and prevent dilated cardiomyopathy. Journal of Molecular and Cellular Cardiology 2012;53:333–41.	survival compared in αMyHC-Cre, other experiments not specified	to 12 months
Yang J, Bücker S, Jungblut B, Böttger T, Cinnamon Y, Tchorz J, et al. Inhibition of Notch2 by Numb/Numblike controls myocardial compaction in the heart. Cardiovasc Res 2012;96:276–85.	Overexpression model	Embryonic
Liu Y, Korte FS, Moussavi-Harami F, Yu M, Razumova M, Regnier M, et al. Transcription factor CHF1/Hey2 regulates EC coupling and heart failure in mice through regulation of FKBP12.6. Am J Physiol Heart Circ Physiol 2012;302:H1860–70.	fl/fl, fl/+;αMyHC-Cre, WT	12 weeks
Xin M, Kim Y, Sutherland LB, Murakami M, Qi X, McAnally J, et al. Hippo pathway effector Yap promotes cardiac regeneration. PNAS 2013;110:13839–44.	fl/fl or not specified	20 weeks
Hohl M, Wagner M, Reil J-C, Müller S-A, Tauchnitz M, Zimmer AM, et al. HDAC4 controls histone methylation in response to elevated cardiac load. J Clin Invest 2013;123:1359–70.	fl/fl	7-13 weeks
Frantz S, Klaiber M, Baba HA, Oberwinkler H, Völker K, Gaβner B, et al. Stress-dependent dilated cardiomyopathy in mice with cardiomyocyte-restricted inactivation of cyclic GMP-dependent protein kinase I. Eur Heart J 2013;34:1233–44.	fl/fl	8-10 weeks
Lübkemeier I, Requardt RP, Lin X, Sasse P, Andrié R, Schrickel JW, et al. Deletion of the last five C-terminal amino acid residues of connexin43 leads to lethal ventricular arrhythmias in mice without affecting coupling via gap junction channels. Basic Res Cardiol 2013;108:348.	fl/fl or fl/+	Neonatal
Liu S, Zhang Y, Moayeri M, Liu J, Crown D, Fattah RJ, et al. Key tissue targets responsible for anthrax-toxin-induced lethality. Nature 2013;501:63–8.	WT	10 weeks
Reynolds JO, Chiang DY, Wang W, Beavers DL, Dixit SS, Skapura DG, et al. Junctophilin-2 is necessary for T-tubule maturation during mouse heart development. Cardiovasc Res 2013;100:44–53.	mix αMyHC-Cre and WT. Genotypes separated for survival studies	p25
Okamoto R, Li Y, Noma K, Hiroi Y, Liu P-Y, Taniguchi M, et al. FHL2 prevents cardiac hypertrophy in mice with cardiac-specific deletion of ROCK2. Faseb J 2013;27:1439–49.	αMyHC-Cre or fl/fl	8-12 week males
Cotter DG, Schugar RC, Wentz AE, d'Avignon DA, Crawford PA. Successful adaptation to ketosis by mice with tissue-specific deficiency of ketone body oxidation. Am J Physiol Endocrinol Metab 2013;304:E363–74.	fl/fl	p0, 6 weeks

Wu S-P, Cheng C-M, Lanz RB, Wang T, Respress JL, Ather S, et al. Atrial identity is determined by a COUP-TFII regulatory network. Dev Cell 2013;25:417–26.	fl/fl	2 months
Neary MT, Mohun TJ, Breckenridge RA. A mouse model to study the link between hypoxia, long QT interval and sudden infant death syndrome. Dis Model Mech 2013;6:503–7.	αMyHC-Cre or fl/+;αMyHC-Cre	Neonatal
Oakley RH, Ren R, Cruz-Topete D, Bird GS, Myers PH, Boyle MC, et al. Essential role of stress hormone signaling in cardiomyocytes for the prevention of heart disease. PNAS 2013;110:17035–40.	fl/fl	12 months
Roy A, Fields WC, Rocha-Resende C, Resende RR, Guatimosim S, Prado VF, et al. Cardiomyocyte-secreted acetylcholine is required for maintenance of homeostasis in the heart. Faseb J 2013;27:5072–82.	fl/fl	3 months
Wetzel-Strong SE, Li M, Klein KR, Nishikimi T, Caron KM. Epicardial-derived adrenomedullin drives cardiac hyperplasia during embryogenesis. Dev Dyn 2014;243:243–56.	hi/hi (fl/fl)	2 months
Zhang XQ, Tang R, Li L, Szucsik A, Javan H, Saegusa N, et al. Cardiomyocyte-specific p65 NF-κB deletion protects the injured heart by preservation of calcium handling. Am J Physiol Heart Circ Physiol 2013;305:H1089–97.	WT	12 months
Inagawa M, Nakajima K, Makino T, Ogawa S, Kojima M, Ito S, et al. Histone H3 lysine 9 methyltransferases, G9a and GLP are essential for cardiac morphogenesis. Mech Dev 2013;130:519–31.	fl/fl	Embryonic
Ribeiro PAB, Ribeiro JP, Minozzo FC, Pavlov I, Leu NA, Kurosaka S, et al. Contractility of myofibrils from the heart and diaphragm muscles measured with atomic force cantilevers: effects of heart-specific deletion of arginyl-tRNA-protein transferase. Int J Cardiol 2013;168:3564–71.	WT	1 year
Arechederra M, Carmona R, González-Nuñez M, Gutiérrez-Uzquiza A, Bragado P, Cruz-González I, et al. Met signaling in cardiomyocytes is required for normal cardiac function in adult mice. Biochim Biophys Acta 2013;1832:2204–15.	Pool of fl/fl and αMyHC-Cre	3-9 months
Palchevska OL, Balatskii VV, Andrejeva AO. Embryonically induced β-catenin haploinsufficiency attenuates postnatal heart development and causes violation of foetal genes program. Biopolymers and Cell 2013: 29(2):124-130.	Pool of all other genotypes	1, 3, 6 months
Huebener P, Gwak G-Y, Pradere J-P, Quinzii CM, Friedman R, Lin C-S, et al. High-mobility group box 1 is dispensable for autophagy, mitochondrial quality control, and organ function in vivo. Cell Metab 2014;19:539–47.	fl/fl	to 8 months
Hong T, Yang H, Zhang S-S, Cho HC, Kalashnikova M, Sun B, et al. Cardiac BIN1 folds T-tubule membrane, controlling ion flux and limiting arrhythmia. Nat Med 2014;20:624–32.	Pool of fl/fl, WT, αMyHC-Cre and fl/+	8-12 weeks
Makara MA, Curran J, Little SC, Musa H, Polina I, Smith SA, et al. Ankyrin-G coordinates intercalated disc signaling platform to regulate cardiac excitability in vivo. Circulation Research 2014;115:929–38.	WT	9 months
Kageyama Y, Hoshijima M, Seo K, Bedja D, Sysa-Shah P, Andrabi SA, et al. Parkin-independent mitophagy requires Drp1 and maintains the integrity of mammalian heart and brain. Embo J 2014;33:2798–813.	fl/+ and fl/fl	3 months
Watson LJ, Long BW, DeMartino AM, Brittian KR, Readnower RD, Brainard RE, et al. Cardiomyocyte Ogt is essential for postnatal viability. Am J Physiol Heart Circ Physiol 2014;306:H142–53.	fl/+	4-8 months females

Yoshida T, Yamashita M, Horimai C, Hayashi M. Kruppel-like factor 4 protein regulates isoproterenol-induced cardiac hypertrophy by modulating myocardin expression and activity. J Biol Chem 2014;289:26107–18.	fl/fl	11-14 weeks
Kreusser MM, Lehmann LH, Keranov S, Hoting M-O, Oehl U, Kohlhaas M, et al. Cardiac CaM Kinase II genes δ and γ contribute to adverse remodeling but redundantly inhibit calcineurin-induced myocardial hypertrophy. Circulation 2014;130:1262–73.	WT or fl/fl	12 weeks
Zhu W, Zhang W, Shou W, Field LJ. P53 inhibition exacerbates late-stage anthracycline cardiotoxicity. Cardiovasc Res 2014;103:81–9.	fl/fl or fl/+	7 weeks
Hasumi Y, Baba M, Hasumi H, Huang Y, Lang M, Reindorf R, et al. Folliculin (Flcn) inactivation leads to murine cardiac hypertrophy through mTORC1 deregulation. Hum Mol Genet 2014;23:5706–19.	fl/+;αMyHC-Cre	3 weeks
Roh J-I, Cheong C, Sung YH, Lee J, Oh J, Lee BS, et al. Perturbation of NCOA6 leads to dilated cardiomyopathy. Cell Rep 2014;8:991–8.	fl/fl	9 months
Cossette SM, Gastonguay AJ, Bao X, Lerch-Gaggl A, Zhong L, Harmann LM, et al. Sucrose non-fermenting related kinase enzyme is essential for cardiac metabolism. Biol Open 2014;4:48–61.	$Cre^{Negative}$, αMyHC-Cre; fl/+	8-12 months
Lübkemeier I, Bosen F, Kim J-S, Sasse P, Malan D, Fleischmann BK, et al. Human Connexin43E42K Mutation From a Sudden Infant Death Victim Leads to Impaired Ventricular Activation and Neonatal Death in Mice. Circ Cardiovasc Genet 2015;8:21–9.	fl/+	Embryonic, perinatal
Liu L, Trent CM, Fang X, Son N-H, Jiang H, Blaner WS, et al. Cardiomyocyte-specific loss of diacylglycerol acyltransferase 1 (DGAT1) reproduces the abnormalities in lipids found in severe heart failure. J Biol Chem 2014;289:29881–91.	fl/fl, not shown: αMyHC-Cre and WT	300 days
Wang ER, Jarrah AA, Benard L, Chen J, Schwarzkopf M, Hadri L, et al. Deletion of CXCR4 in cardiomyocytes exacerbates cardiac dysfunction following isoproterenol administration. Gene Ther 2014;21:496–506.	fl/fl	7-8 weeks
Curran J, Makara MA, Little SC, Musa H, Liu B, Wu X, et al. EHD3-dependent endosome pathway regulates cardiac membrane excitability and physiology. Circulation Research 2014;115:68–78.	WT	8 weeks
Weinreuter M, Kreusser MM, Beckendorf J, Schreiter FC, Leuschner F, Lehmann LH, et al. CaM Kinase II mediates maladaptive post-infarct remodeling and pro-inflammatory chemoattractant signaling but not acute myocardial ischemia/reperfusion injury. EMBO Mol Med 2014;6:1231–45.	FFFF (Double Flox)	12 weeks
Beketaev I, Zhang Y, Kim EY, Yu W, Qian L, Wang J. Critical role of YY1 in cardiac morphogenesis. Dev Dyn 2015: 244(5):669-80.	Not specified	Perinatal
Shen H, Cavallero S, Estrada KD, Sandovici I, Kumar SR, Makita T, et al. Extracardiac control of embryonic cardiomyocyte proliferation and ventricular wall expansion. Cardiovasc Res 2015;105:271–8.	Not specified	Embryonic
Lakhal-Littleton S, Wolna M, Carr CA, Miller JJJ, Christian HC, Ball V, et al. Cardiac ferroportin regulates cellular iron homeostasis and is important for cardiac function. PNAS 2015;112:3164–9.	fl/fl	To 40 weeks
Gonzalez-Valdes I, Hidalgo I, Bujarrabal A, Lara-Pezzi E, Padron-Barthe L, Garcia-Pavia P, et al. Bmi1 limits dilated cardiomyopathy and heart failure by inhibiting cardiac senescence. Nat Commun 2015;6:6473.	fl/fl	25 weeks
Curran J, Musa H, Kline CF, Makara MA, Little SC, Higgins JD, et al. Eps15 Homology Domain-containing Protein 3 Regulates Cardiac T-type Ca2+ Channel Targeting and Function in the Atria. J Biol Chem 2015:jbc.M115.646893.	WT	8-12 weeks

D'Uva G, Aharonov A, Lauriola M, Kain D. ERBB2 triggers mammalian heart regeneration by promoting cardiomyocyte dedifferentiation and proliferation. Nature Cell 2015;17(5):627-38.	Not specified	Embryonic, perinatal

Appendix III. Murine *Lox-like* sites. All sites identified within UCSC genome browser MM10 such that ATNACNNCNTATA NNNTANNN TATANGNNGTNAT (tolerate <= 4 mismatches).

Chromosome	Motif Start Coordinate	Motif Stop Coordinate	Number of Mismatches	Sequence
chr1	4451676	4451710	3	atgactacttattttatattatatatacaattatt
chr1	16633248	16633282	3	attacatgatgtattctatagtatatgaggagata
chr1	26003740	26003774	4	ataactacaaataatataaaatatattggggtaac
chr1	26492405	26492439	4	atatccacatataagtgagtatatattgtgtgagt
chr1	27238638	27238672	4	atcattcaatatagcctaagatatctggggtaaat
chr1	28088038	28088072	4	gtcataacatatataatactatatagggagttcaa
chr1	28322036	28322070	4	atgatctcaaatattgtattatagaagctgtgaat
chr1	28662286	28662320	3	attaataaatatactttaaaatatatgtaatcatt
chr1	29713542	29713576	3	ataacaaagtatatattatcatatatatgattata
chr1	33111464	33111498	4	atgaaaccttatatgttatgttatagctttttaat
chr1	37043652	37043686	3	aatacatcttatatcatattggataggaaataata
chr1	50598895	50598929	4	ataacatcatattaagtacaataaaacctgtaagt
chr1	58793503	58793537	4	ataacaacaaataatgtacaatatcttttgttaaa
chr1	59808601	59808635	3	atttcttcatatatctaagaatatatgaagaaatc
chr1	61895496	61895530	3	atgacatggttgaacctaagttataagttgtcata
chr1	67842181	67842215	4	ataaagtccaatattgtaggttatagaatgttacc
chr1	69317622	69317656	4	ataaccacttatcagtgagtatatatcatgtgagt
chr1	69326206	69326240	3	atcatatcatatatcatatcatatatcatatcata
chr1	69326242	69326276	3	atcatatcatatatcatatcatatatcatatcata
chr1	91647917	91647951	4	atcaccacaaataacataaaatacttggagtaagt
chr1	94850437	94850471	4	ataatagcatacaatataatctatatcaagtaagt
chr1	98426041	98426075	4	atgactttctatagtctattcaatatagagtaaaa
chr1	102952245	102952279	4	ataacacattatattatataatatatgattgcaat
chr1	104125837	104125871	4	ataactacttattagtgagtgtatatcaagtgagt
chr1	111225013	111225047	4	atatccacttataagtaaatgtatatgctgtatgt
chr1	111590036	111590070	3	attacttcatattatatatgctcaatgttgtcatt
chr1	123812730	123812764	3	gtgactccatttaataaatcatataagttgtaatg
chr1	124138567	124138601	4	ataatttcttataaaatacaatatatgtattgtgt
chr1	124407566	124407600	3	tttagatcatatatgatattatatataaagttatc
chr1	125957898	125957932	3	atgatgtgatatatgatatgatatatgatgccata
chr1	128559007	128559041	4	attacatcatataatatattctagttgcagttgcc
chr1	139637585	139637619	4	ataatactctatacagtaagttatactttgttact
chr1	140368951	140368985	4	attacttcctataattaacattataaggcttagca
chr1	141471237	141471271	4	gtgacctactatactatattctatagaaggtcaaa
chr1	141517287	141517321	3	aggacatcatatgtactaacttataagtggatatt
chr1	148926443	148926477	3	attataccatatatcttagacaatatttagtaata
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chr1	170353685	170353719	3	atgtcttcttataatttcatatatatgctgtgcta
chr1	188352247	188352281	4	attattacatatataatatattataaattattaca
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chr2	40745330	40745364	3	ataacagcatatattatagagtatatatatatata
chr2	42276911	42276945	3	atgcttacttataatatacaatatatgttataata
chr2	43146692	43146726	3	attacatactatagaataatataaattaagtcatg
chr2	43463353	43463387	4	attattgcatatataataacatatatggctataaa
chr2	43869144	43869178	4	atgtacacatataaaatagtttaaatggagtcact
chr2	46321581	46321615	4	ataacatcatatatgttctgttttaggcattcaga
chr2	46637913	46637947	3	atatgtgctaatatgctataatatatgtggtaata
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chr2	61419292	61419326	3	atgacaccttattcctaagactatcagtggtcatg
chr2	65189068	65189102	3	aatacagtatataagatataatataaaatgttatg

chr2	66524902	66524936	4	ttgacaacatatagcctagttaagatgaggttaaa
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chr2	67416513	67416547	3	atgacatcttataaactatgatatataaagagacttc
chr2	69196808	69196842	4	atgactacatattggttattttacataaagtaacc
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Bibliography

Abel, E.D., Kaulbach, H.C., Tian, R., Hopkins, J.C.A., Duffy, J., Doetschman, T., Minnemann, T., Boers, M.-E., Hadro, E., Oberste-Berghaus, C., et al. (1999). Cardiac hypertrophy with preserved contractile function after selective deletion of GLUT4 from the heart. Journal of Clinical Investigation *104*, 1703-1714.

Agah, R., Frenkel, P.A., French, B.A., Michael, L.H., Overbeek, P.A., and Schneider, M.D. (1997). Gene recombination in postmitotic cells. Targeted expression of Cre recombinase provokes cardiac-restricted, site-specific rearrangement in adult ventricular muscle in vivo. Journal of Clinical Investigation *100*, 169-179.

Aho, A.V., and Corasick, M.J. (1975). Efficient string matching: an aid to bibliographic search. Commun. ACM *18*, 333-340.

Allen, D.L., Harrison, B.C., Maass, A., Bell, M.L., Byrnes, W.C., and Leinwand, L.A. (2001). Cardiac and skeletal muscle adaptations to voluntary wheel running in the mouse. Journal of Applied Physiology *90*, 1900-1908.

Babiker, F.A., Lips, D., Meyer, R., Delvaux, E., Zandberg, P., Janssen, B., van Eys, G., Grohe, C., and Doevendans, P.A. (2006). Estrogen receptor beta protects the murine heart against left ventricular hypertrophy. Arteriosclerosis, Thrombosis, and Vascular Biology *26*, 1524-1530.

Babiker, F.A., Lips, D.J., Delvaux, E., Zandberg, P., Janssen, B.J., Prinzen, F., van Eys, G., Grohe, C., and Doevendans, P.A. (2007). Oestrogen modulates cardiac ischaemic remodelling through oestrogen receptor-specific mechanisms. Acta Physiol (Oxf) *189*, 23-31.

Banerjee, I., Fuseler, J.W., Price, R.L., Borg, T.K., and Baudino, T.A. (2007). Determination of cell types and numbers during cardiac development in the neonatal and adult rat and mouse. American Journal of Physiology. Heart and Circulatory Physiology *293*, H1883-1891.

Barrick, C.J., Dong, A., Waikel, R., Corn, D., Yang, F., Threadgill, D.W., and Smyth, S.S. (2009). Parent-of-origin effects on cardiac response to pressure overload in mice. AJP: Heart and Circulatory Physiology *297*, H1003-H1009.

Batistuzzo de Medeiros, S.R., Krey, G., Hihi, A.K., and Wahli, W. (1997). Functional interactions between the estrogen receptor and the transcription activator Sp1 regulate the estrogen-dependent transcriptional activity of the vitellogenin A1 io promoter. The Journal of Biological Chemistry *272*, 18250-18260.

Beato, M. (1989). Gene regulation by steroid hormones. Cell *56*, 335-344.

Beato, M., Herrlich, P., and Schutz, G. (1995). Steroid hormone receptors: many actors in search of a plot. Cell *83*, 851-857.

Berg, J.M. (1989). DNA binding specificity of steroid receptors. Cell *57*, 1065-1068.

Bergmann, O., Bhardwaj, R.D., Bernard, S., Zdunek, S., Barnabe-Heider, F., Walsh, S., Zupicich, J., Alkass, K., Buchholz, B.A., Druid, H., et al. (2009). Evidence for cardiomyocyte renewal in humans. Science *324*, 98-102.

Bergo, M.O., Lieu, H.D., Gavino, B.J., Ambroziak, P., Otto, J.C., Casey, P.J., Walker, Q.M., and Young, S.G. (2003). On the Physiological Importance of Endoproteolysis of CAAX Proteins: Heart-speciic Rce1 knockout mice develop a lethal cardiomyopathy. Journal of Biological Chemistry *279*, 4729-4736.

Bernardo, B.C., Weeks, K.L., Pretorius, L., and McMullen, J.R. (2010). Molecular distinction between physiological and pathological cardiac hypertrophy: experimental findings and therapeutic strategies. Pharmacology & Therapeutics *128*, 191-227.

Bernelot Moens, S.J., Schnitzler, G.R., Nickerson, M., Guo, H., Ueda, K., Lu, Q., Aronovitz, M.J., Nickerson, H., Baur, W.E., Hansen, U., et al. (2012). Rapid estrogen receptor signaling is essential for the protective effects of estrogen against vascular injury. Circulation *126*, 1993-2004.

Bersell, K., Choudhury, S., Mollova, M., Polizzotti, B.D., Ganapathy, B., Walsh, S., Wadugu, B., Arab, S., and Kuhn, B. (2013). Moderate and high amounts of tamoxifen in MHC-MerCreMer mice induce a DNA damage response, leading to heart failure and death. Disease Models & Mechanisms *6*, 1459-1469.

Bhandary, B., and Robbins, J. (2015). Giving credence to controls: Avoiding the false phenotype. Journal of molecular and cellular cardiology *86*, 136-137.

Booth, E.A., Obeid, N.R., and Lucchesi, B.R. (2005). Activation of estrogen receptor-alpha protects the in vivo rabbit heart from ischemia-reperfusion injury. American journal of physiology. Heart and Circulatory Physiology *289*, H2039-2047.

Branda, C.S., and Dymecki, S.M. (2004). Talking about a Revolution. Developmental Cell *6*, 7-28.

Brower, G.L., Gardner, J.D., Forman, M.F., Murray, D.B., Voloshenyuk, T., Levick, S.P., and Janicki, J.S. (2006). The relationship between myocardial extracellular matrix remodeling and ventricular function. European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery *30*, 604-610.

Buerger, A., Rozhitskaya, O., Sherwood, M.C., Dorfman, A.L., Bisping, E., Abel, E.D., Pu, W.T., Izumo, S., and Jay, P.Y. (2006). Dilated cardiomyopathy resulting from high-level myocardial expression of Cre-recombinase. Journal of Cardiac Failure *12*, 392-398.

Bush, E., Fielitz, J., Melvin, L., Martinez-Arnold, M., McKinsey, T.A., Plichta, R., and Olson, E.N. (2004). A small molecular activator of cardiac hypertrophy uncovered in a chemical screen for modifiers of the calcineurin signaling pathway. Proceedings of the National Academy of Sciences *101*, 2870-2875.

Cavasin, M.A., Tao, Z., Menon, S., and Yang, X.-P. (2004). Gender differences in cardiac function during early remodeling after acute myocardial infarction in mice. Life Sciences *75*, 2181-2192.

Chen, J.Q., Delannoy, M., Cooke, C., and Yager, J.D. (2004). Mitochondrial localization of ERalpha and ERbeta in human MCF7 cells. American Journal of Physiology. Endocrinology and Metabolism *286*, E1011-1022.

Chung, E., and Leinwand, L.A. (2014). Pregnancy as a cardiac stress model. Cardiovascular Research *101*, 561-570.

Clerk, A., and Sugden, P. (2006). Inflame my heart (by p38-MAPK). Circulation Research *99*, 455-458.

Cosper, P.F., Harvey, P.A., and Leinwand, L.A. (2012). Interferon-gamma causes cardiac myocyte atrophy via selective degradation of myosin heavy chain in a model of chronic myocarditis. The American Journal of Pathology *181*, 2038-2046.

Couse, J.F., Curtis, S.W., Washburn, T.F., Lindzey, J., Golding, T.S., Lubahn, D.B., Smithies, O., and Korach, K.S. (1995). Analysis of transcription and estrogen insensitivity in the female mouse after targeted disruption of the estrogen receptor gene. Mol Endocrinol *9*, 1441-1454.

Crawford, D.C., Chobanian, A.V., and Brecher, P. (1994). Angiotensin II induces fibronectin expression associated with cardiac fibrosis in the rat. Circulation Research *74*, 727-739.

Davis, J., Maillet, M., Miano, J.M., and Molkentin, J.D. (2012). Lost in transgenesis: A user's guide for genetically manipulating the mouse in cardiac research. Circulation Research *111*, 761-777.

Devanathan, S., Whitehead, T., Schweitzer, G.G., Fettig, N., Kovacs, A., Korach, K.S., Finck, B.N., and Shoghi, K.I. (2014). An animal model with a cardiomyocyte-specific deletion of estrogen receptor alpha: functional, metabolic, and differential network analysis. PloS One *9*, e101900.

Doetschman, T., and Azhar, M. (2012). Cardiac-specific inducible and conditional gene targeting in mice. Circulation Research *110*, 1498-1512.

Douglas, P.S., Katz, S.E., Weinberg, E.O., Chen, M.H., Bishop, S.P., and Lorell, B.H. (1998). Hypertrophic remodeling: gender differences in the early response to left ventricular pressure overload. Journal of the American College of Cardiology *32*, 1118-1125.

Dupont, S., Krust, A., Gansmuller, A., Dierich, A., Chambon, P., and Mark, M. (2000). Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. Development *127*, 4277-4291.

El-Tanani, M.K., and Green, C.D. (1997). Two separate mechanisms for ligand-independent activation of the estrogen receptor. Mol Endocrinol *11*, 928-937.

Fabiato, A. (1983). Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. The American Journal of Physiology *245*, C1-14.

Farach-Carson, M.C., and Davis, P.J. (2003). Steroid hormone interactions with target cells: cross talk between membrane and nuclear pathways. The Journal of Pharmacology and Experimental Therapeutics *307*, 839-845.

Feil, R., Wagner, J., Metzger, D., and Chambon, P. (1997). Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. Biochemical and Biophysical Research Communications *237*, 752-757.

Figtree, G.A., McDonald, D., Watkins, H., and Channon, K.M. (2003). Truncated estrogen receptor alpha 46-kDa isoform in human endothelial cells: relationship to acute activation of nitric oxide synthase. Circulation *107*, 120-126.

Flouriot, G., Brand, H., Denger, S., Metivier, R., Kos, M., Reid, G., Sonntag-Buck, V., and Gannon, F. (2000). Identification of a new isoform of the human estrogen receptor-alpha (hER-alpha) that is encoded by distinct transcripts and that is able to repress hER-alpha activation function 1. The EMBO Journal *19*, 4688-4700.

Flouriot, G., Griffin, C., Kenealy, M., Sonntag-Buck, V., and Gannon, F. (1998). Differentially expressed messenger RNA isoforms of the human estrogen receptor-alpha gene are generated by alternative splicing and promoter usage. Mol Endocrinol *12*, 1939-1954.

Frey, N., McKinsey, T.A., and Olson, E.N. (2000). Decoding calcium signals involved in cardiac growth and function. Nature Medicine *6*, 1221-1227.

Galien, R., and Garcia, T. (1997). Estrogen receptor impairs interleukin-6 expression by preventing protein binding on the NF-kappaB site. Nucleic Acids Res *25*, 2424-2429.

Geraldes, P., Sirois, M.G., and Tanguay, J.F. (2003). Specific contribution of estrogen receptors on mitogen-activated protein kinase pathways and vascular cell activation. Circulation Research *93*, 399-405.

Gillies, G.E., and McArthur, S. (2010). Estrogen actions in the brain and the basis for differential action in men and women: a case for sex-specific medicines. Pharmacological Reviews *62*, 155-198.

Greene, G.L., Gilna, P., Waterfield, M., Baker, A., Hort, Y., and Shine, J. (1986). Sequence and expression of human estrogen receptor complementary DNA. Science *231*, 1150-1154.

Grimm, D., Elsner, D., Schunkert, H., Pfeifer, M., Griese, D., Bruckschlegel, G., Muders, F., Riegger, G.A., and Kromer, E.P. (1998). Development of heart failure following

isoproterenol administration in the rat: role of the renin-angiotensin system. Cardiovascular Research *37*, 91-100.

Guan, Z., Baier, L.D., and Morrison, A.R. (1997). p38 Mitogen-activated protein kinase down-regulates Nitric Oxide and up-regulates prostaglandin E2 biosynthesis stimulated by Interleukin-1. Journal of Biological Chemistry *272*, 8083-8089.

Guo, F., Gopaul, D.N., and van Duyne, G.D. (1997). Structure of Cre recombinase complexed with DNA in a site-specific recombination synapse. Nature *389*, 40-46.

Haines, C., Harvey, P., and Leinwand, L.A. (2012a). Estrogens mediate cardiac hypertrophy in a stimulus-dependent manner. Endocrinology *153*, 4480-4490.

Haines, C.D., Harvey, P.A., Luczak, E.D., Barthel, K.K., Konhilas, J.P., Watson, P.A., Stauffer, B.L., and Leinwand, L.A. (2012b). Estrogenic compounds are not always cardioprotective and can be lethal in males with genetic heart disease. Endocrinology *153*, 4470-4479.

Hall, J.M., Couse, J.F., and Korach, K.S. (2001). The multifaceted mechanisms of estradiol and estrogen receptor signaling. The Journal of Biological Chemistry *276*, 36869-36872.

Harary, I., and Farley, B. (1960). In vitro studies of single isolated beating heart cells. Science *131*, 1674-1675.

Harno, E., Cottrell, Elizabeth C., and White, A. (2013). Metabolic pitfalls of CNS Cre-based technology. Cell Metabolism *18*, 21-28.

Harvey, P.A., and Leinwand, L.A. (2011). The cell biology of disease: cellular mechanisms of cardiomyopathy. J Cell Biol *194*, 355-365.

Hatano, A., Okada, J., Hisada, T., and Sugiura, S. (2012). Critical role of cardiac t-tubule system for the maintenance of contractile function revealed by a 3D integrated model of cardiomyocytes. Journal of Biomechanics *45*, 815-823.

Higgins, C.B., Vatner, S.F., Franklin, D., and Braunwald, E. (1973). Extent of regulation of the heart's contractile state in the conscious dog by alteration in the frequency of contraction. Journal of Clinical Investigation *52*, 1187-1194.

Hoess, R.H., Wierzbicki, A., and Abremski, K. (1986). The role of the loxP spacer region in PI site-specific recombination. Nucl Acids Res *14*, 2287-2300.

Iovino, N., Denti, M.A., Bozzoni, I., and Cortese, R. (2005). A loxP-Containing pol II Promoter for RNA Interference is Reversibly Regulated by Cre Recombinase. RNA Biology *2*, 86-92.

Irsik, D.L., Carmines, P.K., and Lane, P.H. (2013). Classical estrogen receptors and ERalpha splice variants in the mouse. PloS One *8*, e70926.

Ito, M., Yamanouchi, K., Naito, K., Calos, M.P., and Tojo, H. (2010). Site-specific integration of transgene targeting an endogenous lox-like site in early mouse embryos. J Appl Genetics *52*, 89-94.

Itoh, G., Tamura, J., Suzuki, M., Suzuki, Y., Ikeda, H., Koike, M., Nomura, M., Jie, T., and Ito, K. (1995). DNA fragmentation of human infarcted myocardial cells demonstrated by the nick end labeling method and DNA agarose gel electrophoresis. The American Journal of Pathology *146*, 1325-1331.

Jae Huh, W., Mysorekar, I.U., and Mills, J.C. (2010). Inducible activation of Cre recombinase in adult mice causes gastric epithelial atrophy, metaplasia, and regenerative changes in the absence of "floxed" alleles. AJP: Gastrointestinal and Liver Physiology *299*, G368-G380.

Janbandhu, V., Moik, D., and Fässler, R. (2013). Cre recombinase induces DNA damage and tetraploidy in the absence of LoxP sites. Cell Cycle *13*, 462-470.

Jayachandran, M., Preston, C.C., Hunter, L.W., Jahangir, A., Owen, W.G., Korach, K.S., and Miller, V.M. (2010). Loss of estrogen receptor beta decreases mitochondrial energetic potential and increases thrombogenicity of platelets in aged female mice. Age (Dordr) *32*, 109-121.

Jin, H., Yang, R., Li, W., Lu, H., Ryan, A.M., Ogasawara, A.K., Van Peborgh, J., and Paoni, N.F. (2000). Effects of exercise training on cardiac function, gene expression, and apoptosis in rats. American journal of physiology. Heart and Circulatory Physiology *279*, H2994-3002.

Joshi, M., Keith Pittman, H., Haisch, C., and Verbanac, K. (2008). Real-time PCR to determine transgene copy number and to quantitate the biolocalization of adoptively transferred cells from EGFP-transgenic mice. BioTechniques *45*, 247-258.

Kamo, T., Akazawa, H., and Komuro, I. (2015). Cardiac nonmyocytes in the hub of cardiac hypertrophy. Circulation Research *117*, 89-98.

Kang, L., Zhang, X., Xie, Y., Tu, Y., Wang, D., Liu, Z., and Wang, Z.Y. (2010). Involvement of estrogen receptor variant ER-alpha36, not GPR30, in nongenomic estrogen signaling. Mol Endocrinol *24*, 709-721.

Kastner, P., Krust, A., Mendelsohn, C., Garnier, J.M., Zelent, A., Leroy, P., Staub, A., and Chambon, P. (1990). Murine isoforms of retinoic acid receptor gamma with specific patterns of expression. Proceedings of the National Academy of Sciences of the United States of America *87*, 2700-2704.

Kim, K.H., Toomre, D., and Bender, J.R. (2011). Splice isoform estrogen receptors as integral transmembrane proteins. Molecular Biology of the Cell *22*, 4415-4423.

Koitabashi, N., Bedja, D., Zaiman, A.L., Pinto, Y.M., Zhang, M., Gabrielson, K.L., Takimoto, E., and Kass, D.A. (2009). Avoidance of transient cardiomyopathy in

cardiomyocyte-targeted tamoxifen-induced MerCreMer gene deletion models. Circulation Research *105*, 12-15.

Konhilas, J.P., Maass, A.H., Luckey, S.W., Stauffer, B.L., Olson, E.N., and Leinwand, L.A. (2004). Sex modifies exercise and cardiac adaptation in mice. American journal of physiology. Heart and Circulatory Physiology *287*, H2768-2776.

Konhilas, J.P., Watson, P.A., Maass, A., Boucek, D.M., Horn, T., Stauffer, B.L., Luckey, S.W., Rosenberg, P., and Leinwand, L.A. (2006). Exercise Can Prevent and Reverse the Severity of Hypertrophic Cardiomyopathy. Circulation Research *98*, 540-548.

Kumar, R., and Thompson, E.B. (1999). The structure of the nuclear hormone receptors. Steroids *64*, 310-319.

Kwon, C., Arnold, J., Hsiao, E.C., Taketo, M.M., Conklin, B.R., and Srivastava, D. (2007). Canonical Wnt signaling is a positive regulator of mammalian cardiac progenitors. Proceedings of the National Academy of Sciences of the United States of America *104*, 10894-10899.

Lee, L.M., Cao, J., Deng, H., Chen, P., Gatalica, Z., and Wang, Z.Y. (2008). ER-alpha36, a novel variant of ER-alpha, is expressed in ER-positive and -negative human breast carcinomas. Anticancer Res *28*, 479-483.

Lehman, W., Craig, R., and Vibert, P. (1994). Ca(2+)-induced tropomyosin movement in Limulus thin filaments revealed by three-dimensional reconstruction. Nature *368*, 65-67.

Lexow, J., Poggioli, T., Sarathchandra, P., Santini, M.P., and Rosenthal, N. (2013). Cardiac fibrosis in mice expressing an inducible myocardial-specific Cre driver. Disease Models & Mechanisms *6*, 1470-1476.

Li, F., Wang, X., Capasso, J.M., and Gerdes, A.M. (1996). Rapid transition of cardiac myocytes from hyperplasia to hypertrophy during postnatal development. Journal of Molecular and Cellular Cardiology *28*, 1737-1746.

Li, L., Haynes, M.P., and Bender, J.R. (2003). Plasma membrane localization and function of the estrogen receptor alpha variant (ER46) in human endothelial cells. Proceedings of the National Academy of Sciences of the United States of America *100*, 4807-4812.

Lindner, V., Kim, S.K., Karas, R.H., Kuiper, G.G., Gustafsson, J.A., and Mendelsohn, M.E. (1998). Increased expression of estrogen receptor-beta mRNA in male blood vessels after vascular injury. Circulation Research *83*, 224-229.

Liu, Y.-G., and Whittier, R.F. (1995). Thermal asymmetric interlaced PCR: automatable amplification and sequencing of insert end fragments from P1 and YAC clones for chromosome walking. Genomics *25*, 674-681.

Lizotte, E., Grandy, S.A., Tremblay, A., Allen, B.G., and Fiset, C. (2009). Expression, distribution and regulation of sex steroid hormone receptors in mouse heart. Cellular Physiology and Biochemistry : International Journal of Experimental Cellular Physiology, Biochemistry, and Pharmacology *23*, 75-86.

Lu, Q., Pallas, D.C., Surks, H.K., Baur, W.E., Mendelsohn, M.E., and Karas, R.H. (2004). Striatin assembles a membrane signaling complex necessary for rapid, nongenomic activation of endothelial NO synthase by estrogen receptor alpha. Proceedings of the National Academy of Sciences of the United States of America *101*, 17126-17131.

Lubahn, D.B., Moyer, J.S., Golding, T.S., Couse, J.F., Korach, K.S., and Smithies, O. (1993). Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proceedings of the National Academy of Sciences of the United States of America *90*, 11162-11166.

Luczak, E.D., and Leinwand, L.A. (2009). Sex-Based Cardiac Physiology. Annu. Rev. Physiol. *71*, 1-18.

Maass, A.H., and Buvoli, M. (2007). Cardiomyocyte preparation, culture, and gene transfer. Methods Mol Biol *366*, 321-330.

Mahdavi, V., Lompre, A.M., Chambers, A.P., and Nadal-Ginard, B. (1984). Cardiac myosin heavy chain isozymic transitions during development and under pathological conditions are regulated at the level of mRNA availability. European Heart Journal *5 Suppl F*, 181-191.

Mahmoodzadeh, S., Eder, S., Nordmeyer, J., Ehler, E., Huber, O., Martus, P., Weiske, J., Pregla, R., Hetzer, R., and Regitz-Zagrosek, V. (2006). Estrogen receptor alpha up-regulation and redistribution in human heart failure. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *20*, 926-934.

Maillet, M., Davis, J., Auger-Messier, M., York, A., Osinska, H., Piquereau, J., Lorenz, J.N., Robbins, J., Ventura-Clapier, R., and Molkentin, J.D. (2010). Heart-specific deletion of CnB1 reveals multiple mechanisms whereby calcineurin regulates cardiac growth and function. The Journal of Biological Chemistry *285*, 6716-6724.

Marquez, D.C., and Pietras, R.J. (2001). Membrane-associated binding sites for estrogen contribute to growth regulation of human breast cancer cells. Oncogene *20*, 5420-5430.

Matkovich, S.J., Zhang, Y., Van Booven, D.J., and Dorn, G.W. (2010). Deep mRNA sequencing for in vivo functional analysis of cardiac transcriptional regulators: application to Gαq. Circulation Research *106*, 1459-1467.

Matsui, T., Li, L., Wu, J.C., Cook, S.A., Nagoshi, T., Picard, M.H., Liao, R., and Rosenzweig, A. (2002). Phenotypic spectrum caused by transgenic overexpression of activated Akt in the heart. The Journal of Biological Chemistry *277*, 22896-22901.

Matsui, T., Tao, J., del Monte, F., Lee, K.H., Li, L., Picard, M., Force, T.L., Franke, T.F., Hajjar, R.J., and Rosenzweig, A. (2001). Akt activation preserves cardiac function and prevents injury after transient cardiac ischemia in vivo. Circulation *104*, 330-335.

McFadden, D.G., Barbosa, A.C., Richardson, J.A., Schneider, M.D., Srivastava, D., and Olson, E.N. (2005). The Hand1 and Hand2 transcription factors regulate expansion of the embryonic cardiac ventricles in a gene dosage-dependent manner. Development *132*, 189-201.

McInerney, E.M., and Katzenellenbogen, B.S. (1996). Different regions in activation function-1 of the human estrogen receptor required for antiestrogen- and estradiol-dependent transcription activation. The Journal of Biological Chemistry *271*, 24172-24178.

McKee, P.A., Castelli, W.P., McNamara, P.M., and Kannel, W.B. (1971). The natural history of congestive heart failure: the Framingham study. The New England Journal of Medicine *285*, 1441-1446.

McMullen, J.R., Amirahmadi, F., Woodcock, E.A., Schinke-Braun, M., Bouwman, R.D., Hewitt, K.A., Mollica, J.P., Zhang, L., Zhang, Y., Shioi, T., et al. (2007). Protective effects of exercise and phosphoinositide 3-kinase(p110alpha) signaling in dilated and hypertrophic cardiomyopathy. Proceedings of the National Academy of Sciences of the United States of America *104*, 612-617.

McMullen, J.R., Shioi, T., Huang, W.Y., Zhang, L., Tarnavski, O., Bisping, E., Schinke, M., Kong, S., Sherwood, M.C., Brown, J., et al. (2004). The insulin-like growth factor 1 receptor induces physiological heart growth via the phosphoinositide 3-kinase(p110alpha) pathway. The Journal of biological chemistry *279*, 4782-4793.

McMullen, J.R., Shioi, T., Zhang, L., Tarnavski, O., Sherwood, M.C., Kang, P.M., and Izumo, S. (2003). Phosphoinositide 3-kinase(p110alpha) plays a critical role for the induction of physiological, but not pathological, cardiac hypertrophy. Proceedings of the National Academy of Sciences of the United States of America *100*, 12355-12360.

Mendelsohn, M.E., and Karas, R.H. (2005). Molecular and cellular basis of cardiovascular gender differences. Science *308*, 1583-1587.

Molkentin, J.D., Lu, J.-R., Antos, C.L., Markham, B., Richardson, J., Robbins, J., Grant, S.R., and Olson, E.N. (1998). A Calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell *93*, 215-228.

Molkentin, J.D., and Robbins, J. (2009). With great power comes great responsibility: Using mouse genetics to study cardiac hypertrophy and failure. Journal of Molecular and Cellular Cardiology *46*, 130-136.

Morani, A., Barros, R.P., Imamov, O., Hultenby, K., Arner, A., Warner, M., and Gustafsson, J.A. (2006). Lung dysfunction causes systemic hypoxia in estrogen receptor

beta knockout (ERbeta-/-) mice. Proceedings of the National Academy of Sciences of the United States of America *103*, 7165-7169.

Mozaffarian, D., Benjamin, E.J., Go, A.S., Arnett, D.K., Blaha, M.J., Cushman, M., de Ferranti, S., Despres, J.P., Fullerton, H.J., Howard, V.J., et al. (2015). Heart disease and stroke statistics--2015 update: a report from the American Heart Association. Circulation *131*, e29-322.

Nuedling, S., Kahlert, S., Loebbert, K., Doevendans, P.A., Meyer, R., Vetter, H., and Grohe, C. (1999a). 17 Beta-estradiol stimulates expression of endothelial and inducible NO synthase in rat myocardium in-vitro and in-vivo. Cardiovascular Research *43*, 666-674.

Nuedling, S., Kahlert, S., Loebbert, K., Meyer, R., Vetter, H., and Grohe, C. (1999b). Differential effects of 17beta-estradiol on mitogen-activated protein kinase pathways in rat cardiomyocytes. FEBS letters *454*, 271-276.

Ohlsson, C., Hellberg, N., Parini, P., Vidal, O., Bohlooly, Y.M., Rudling, M., Lindberg, M.K., Warner, M., Angelin, B., and Gustafsson, J.A. (2000). Obesity and disturbed lipoprotein profile in estrogen receptor-alpha-deficient male mice. Biochemical and Biophysical Research Communications *278*, 640-645.

Ohshiro, K., Schwartz, A.M., Levine, P.H., and Kumar, R. (2012). Alternate estrogen receptors promote invasion of inflammatory breast cancer cells via non-genomic signaling. PloS One *7*, e30725.

Oka, T., Maillet, M., Watt, A.J., Schwartz, R.J., Aronow, B.J., Duncan, S.A., and Molkentin, J.D. (2006). Cardiac-specific deletion of Gata4 reveals its requirement for hypertrophy, compensation, and myocyte viability. Circulation Research *98*, 837-845.

Osadchii, O.E. (2007). Cardiac hypertrophy induced by sustained beta-adrenoreceptor activation: pathophysiological aspects. Heart Failure Reviews *12*, 66-86.

Pare, G., Krust, A., Karas, R.H., Dupont, S., Aronovitz, M., Chambon, P., and Mendelsohn, M.E. (2002). Estrogen receptor-alpha mediates the protective effects of estrogen against vascular injury. Circulation Research *90*, 1087-1092.

Patten, R.D., and Karas, R.H. (2006). Estrogen replacement and cardiomyocyte protection. Trends in Cardiovascular Medicine *16*, 69-75.

Patten, R.D., Pourati, I., Aronovitz, M.J., Baur, J., Celestin, F., Chen, X., Michael, A., Haq, S., Nuedling, S., Grohe, C., et al. (2004). 17beta-estradiol reduces cardiomyocyte apoptosis in vivo and in vitro via activation of phospho-inositide-3 kinase/Akt signaling. Circulation Research *95*, 692-699.

Pedram, A., Razandi, M., O'Mahony, F., Lubahn, D., and Levin, E.R. (2010). Estrogen receptor-beta prevents cardiac fibrosis. Mol Endocrinol *24*, 2152-2165.

Pelzer, T., Loza, P.A., Hu, K., Bayer, B., Dienesch, C., Calvillo, L., Couse, J.F., Korach, K.S., Neyses, L., and Ertl, G. (2005). Increased mortality and aggravation of heart failure in estrogen receptor-beta knockout mice after myocardial infarction. Circulation *111*, 1492-1498.

Petz, L.N., Ziegler, Y.S., Schultz, J.R., Kim, H., Kemper, J.K., and Nardulli, A.M. (2004). Differential regulation of the human progesterone receptor gene through an estrogen response element half site and Sp1 sites. The Journal of Steroid Biochemistry and Molecular Biology *88*, 113-122.

Pierce, K.L., Tohgo, A., Ahn, S., Field, M.E., Luttrell, L.M., and Lefkowitz, R.J. (2001). Epidermal growth factor (EGF) receptor-dependent ERK activation by G protein-coupled receptors: a co-culture system for identifying intermediates upstream and downstream of heparin-binding EGF shedding. The Journal of Biological Chemistry *276*, 23155-23160.

Pillai, M.M., Venkataraman, G.M., Kosak, S., and Torok-Storb, B. (2007). Integration site analysis in transgenic mice by thermal asymmetric interlaced (TAIL)-PCR: segregating multiple-integrant founder lines and determining zygosity. Transgenic Res *17*, 749-754.

Pugach, E.K., Richmond, P.A., Azofeifa, J.G., Dowell, R.D., and Leinwand, L.A. (2015). Prolonged Cre expression driven by the alpha-myosin heavy chain promoter can be cardiotoxic. Journal of Molecular and Cellular Cardiology *86*, 54-61.

Qiu, Z., Cang, Y., and Goff, S.P. (2009). c-Abl tyrosine kinase regulates cardiac growth and development. Proceedings of the National Academy of Sciences *107*, 1136-1141.

Quinlan, A.R., and Hall, I.M. (2010). BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics *26*, 841-842.

Reecy, J.M., Li, X., Yamada, M., DeMayo, F.J., Newman, C.S., Harvey, R.P., and Schwartz, R.J. (1999). Identification of upstream regulatory regions in the heart-expressed homeobox gene Nkx2-5. Development *126*, 839-849.

Resnicow, D.I., Deacon, J.C., Warrick, H.M., Spudich, J.A., and Leinwand, L.A. (2010). Functional diversity among a family of human skeletal muscle myosin motors. Proceedings of the National Academy of Sciences of the United States of America *107*, 1053-1058.

Ropero, A.B., Eghbali, M., Minosyan, T.Y., Tang, G., Toro, L., and Stefani, E. (2006). Heart estrogen receptor alpha: distinct membrane and nuclear distribution patterns and regulation by estrogen. Journal of Molecular and Cellular Cardiology *41*, 496-510.

Rossi, A., Kontarakis, Z., Gerri, C., Nolte, H., Holper, S., Kruger, M., and Stainier, D.Y. (2015). Genetic compensation induced by deleterious mutations but not gene knockdowns. Nature *524*, 230-233.

Rufer, A.W., and Sauer, B. (2002). Non-contact positions impose site selectivity on Cre recombinase. Nucleic Acids Research *30*, 2764-2771.

Russell, B., Motlagh, D., and Ashley, W.W. (2000). Form follows function: how muscle shape is regulated by work. Journal of Applied Physiology *88*, 1127-1132.

Sanford, L.P., Kallapur, S., Ormsby, I., and Doetschman, T. (2001). Influence of Genetic Background on Knockout Mouse Phenotypes. In Gene Knockout Protocols (Springer Science + Business Media), pp. 217-225.

Schaefer, S., Taylor, A.L., Lee, H.R., Niggemann, E.H., Levine, B.D., Popma, J.J., Mitchell, J.H., and Hillis, L.D. (1988). Effect of increasing heart rate on left ventricular performance in patients with normal cardiac function. The American Journal of Cardiology *61*, 617-620.

Schmidt, E.E., Taylor, D.S., Prigge, J.R., Barnett, S., and Capecchi, M.R. (2000). Illegitimate Cre-dependent chromosome rearrangements in transgenic mouse spermatids. Proceedings of the National Academy of Sciences *97*, 13702-13707.

Schulkey, C.E., Regmi, S.D., Magnan, R.A., Danzo, M.T., Luther, H., Hutchinson, A.K., Panzer, A.A., Grady, M.M., Wilson, D.B., and Jay, P.Y. (2015). The maternal-age-associated risk of congenital heart disease is modifiable. Nature *520*, 230-233.

Schulz, K.U., and Mihov, S. (2002). Fast string correction with Levenshtein automata. International Journal on Document Analysis and Recognition *5*, 67-85.

Semsarian, C., Healey, M.J., Fatkin, D., Giewat, M., Duffy, C., Seidman, C.E., and Seidman, J.G. (2001). A polymorphic modifier gene alters the hypertrophic response in a murine model of familial hypertrophic cardiomyopathy. Journal of Molecular and Cellular Cardiology *33*, 2055-2060.

Simoncini, T., Hafezi-Moghadam, A., Brazil, D.P., Ley, K., Chin, W.W., and Liao, J.K. (2000). Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. Nature *407*, 538-541.

Simpson, P., McGrath, A., and Savion, S. (1982). Myocyte hypertrophy in neonatal rat heart cultures and its regulation by serum and by catecholamines. Circulation Research *51*, 787-801.

Skavdahl, M., Steenbergen, C., Clark, J., Myers, P., Demianenko, T., Mao, L., Rockman, H.A., Korach, K.S., and Murphy, E. (2005). Estrogen receptor-beta mediates male-female differences in the development of pressure overload hypertrophy. American Journal of Physiology. Heart and Circulatory Physiology *288*, H469-476.

Sohal, D.S., Nghiem, M., Crackower, M.A., Witt, S.A., Kimball, T.R., Tymitz, K.M., Penninger, J.M., and Molkentin, J.D. (2001). Temporally regulated and tissue-specific gene manipulations in the adult and embryonic heart using a tamoxifen-inducible Cre protein. Circulation Research *89*, 20-25.

Sternberg, N., Hamilton, D., Austin, S., Yarmolinsky, M., and Hoess, R. (1981). Site-specific recombination and its role in the life cycle of bacteriophage P1. Cold Spring Harbor Symposia on Quantitative Biology *45*, 297-309.

Subramaniam, A., Jones, W.K., Gulick, J., Wert, S., Neumann, J., and Robbins, J. (1991). Tissue-specific regulation of the alpha-myosin heavy chain gene promoter in transgenic mice. The Journal of Biological Chemistry *266*, 24613-24620.

Thyagarajan, B., Guimarães, M.J., Groth, A.C., and Calos, M.P. (2000). Mammalian genomes contain active recombinase recognition sites. Gene *244*, 47-54.

Vega, R.B., Horton, J.L., and Kelly, D.P. (2015). Maintaining ancient organelles: mitochondrial biogenesis and maturation. Circulation Research *116*, 1820-1834.

Venkov, C.D., Rankin, A.B., and Vaughan, D.E. (1996). Identification of authentic estrogen receptor in cultured endothelial cells. A potential mechanism for steroid hormone regulation of endothelial function. Circulation *94*, 727-733.

Verrou, C., Zhang, Y., Zurn, C., Schamel, W.W., and Reth, M. (1999). Comparison of the tamoxifen regulated chimeric Cre recombinases MerCreMer and CreMer. Biological chemistry *380*, 1435-1438.

Villarreal, F.J., and Dillmann, W.H. (1992). Cardiac hypertrophy-induced changes in mRNA levels for TGF-beta 1, fibronectin, and collagen. The American Journal of Physiology *262*, H1861-1866.

Wang, Q., Reiter, R.S., Huang, Q.Q., Jin, J.P., and Lin, J.J. (2001). Comparative studies on the expression patterns of three troponin T genes during mouse development. The Anatomical Record *263*, 72-84.

Wang, Z., Zhang, X., Shen, P., Loggie, B.W., Chang, Y., and Deuel, T.F. (2005). Identification, cloning, and expression of human estrogen receptor-alpha36, a novel variant of human estrogen receptor-alpha66. Biochemical and Biophysical Research Communications *336*, 1023-1027.

Wang, Z., Zhang, X., Shen, P., Loggie, B.W., Chang, Y., and Deuel, T.F. (2006). A variant of estrogen receptor-{alpha}, hER-{alpha}36: transduction of estrogen- and antiestrogen-dependent membrane-initiated mitogenic signaling. Proceedings of the National Academy of Sciences of the United States of America *103*, 9063-9068.

Weigel, N.L., and Zhang, Y. (1998). Ligand-independent activation of steroid hormone receptors. J Mol Med (Berl) *76*, 469-479.

Yamamoto, K.R. (1985). Steroid receptor regulated transcription of specific genes and gene networks. Annual review of genetics *19*, 209-252.

Yamazaki, T., Tobe, K., Hoh, E., Maemura, K., Kaida, T., Komuro, I., Tamemoto, H., Kadowaki, T., Nagai, R., and Yazaki, Y. (1993). Mechanical loading activates mitogen-

activated protein kinase and S6 peptide kinase in cultured rat cardiac myocytes. The Journal of Biological Chemistry *268*, 12069-12076.

Zak, R. (1974). Development and proliferative capacity of cardiac muscle cells. Circulation Research *35*, suppl II:17-26.

Zechner, D., Thuerauf, D.J., Hanford, D.S., McDonough, P.M., and Glembotski, C.C. (1997). A Role for the p38 Mitogen-activated Protein Kinase Pathway in Myocardial Cell Growth, Sarcomeric Organization, and Cardiac-specific Gene Expression. The Journal of Cell Biology *139*, 115-127.

Zheng, Y., Zhang, J., Xu, Z.Z., Sheng, J.M., Zhang, X.C., Wang, H.H., Teng, X.D., Liu, X.J., Cao, J., and Teng, L.S. (2010). Quantitative profiles of the mRNAs of ER-alpha and its novel variant ER-alpha36 in breast cancers and matched normal tissues. Journal of Zhejiang University. Science. B *11*, 144-150.

Zhu, Y., Bian, Z., Lu, P., Karas, R.H., Bao, L., Cox, D., Hodgin, J., Shaul, P.W., Thoren, P., Smithies, O., et al. (2002). Abnormal vascular function and hypertension in mice deficient in estrogen receptor beta. Science *295*, 505-508.