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

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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 [1] 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

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Disclosures

None

αMyHC-Cre mice as controls in conditional knockout studies as well as the need for a less cardiotoxic *Cre* driver for the field.

Keywords

Transgenic mice; Cre recombinase; *LoxP* site; DNA damage; Cardiotoxicity

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 [2]). 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.

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 [1,3]. *Cre* expression from this promoter has been shown to be both cardiac myocyte-specific and to drive highly efficient recombination [1]. 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 (Supplemental Table 1) 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.

Cre recombinase mediates recombination between pairs of 34-base-pair palindromic *loxP* sites [4]. Although its preference is for sites consisting of two 13-base-pair inverted repeats separated by an 8-base-pair spacer [5], 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 [6]. Such degenerate *loxP* sites have been identified in mammalian genomes and can serve as legitimate Cre substrates [7]. Further, it has been shown that Cre binding to *loxP* sites in the absence of recombination has the ability to block downstream transcription [8]. 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 [9,10]. Toxic effects associated with Cre expression have been observed in gastrointestinal cells, neurons, and spermatids [10–12]. Finally, tamoxifen-inducible Cre expression in myocardium has also been demonstrated to induce fibrosis, DNA damage, and cardiac dilation independently of tamoxifen-associated toxicity [13–16]. However a *Cre*-only control genotype is not often

included in studies requiring Cre expression. In fact, the αMyHC-Cre$^{+/-}$ genotype is only included as a control group in ~20% of studies identified that utilize this *Cre* line (Supplemental Table 1).

Given the extensive use of the αMyHC-Cre mice and anecdotal reports of associated cardiotoxicity [17–19], 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 αMyHC-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.

2. 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. αMyHC-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 αMyHC-Cre$^{+/-}$ mice. Genotyping was performed from tail snip biopsies and re-confirmed post-mortem. Primers for genotyping are listed in Supplemental Table 2. Myocardial *Cre* expression was confirmed by quantitative real-time reverse transcriptase PCR (qRT-PCR) and western blot for Cre expression (Supplemental Figure 1). For sample collection, animals were sedated using 1–4% inhaled isoflurane and sacrificed by cervical dislocation. Hearts were excised, perfused in 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 (Supplemental Table 2) 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. 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 ([20]), 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 [6] (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 [21]. Thusly, LADMA accepts a number of mismatches (in our case 4) and builds a Levenshtein non-determinisitc 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 [22].

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 (Supplemental Table 4). 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) [23]. 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 [24]. 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 10ng 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 [25,26]. 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 was 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 pairways comparisons. *P* values less than 0.05 were considered significant unless otherwise noted.

3. Results

3.1 α*MyHC-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 [17–19], 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 [1]. 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 (Supplemental Table 1). 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

[27]. 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 1A,B and Supplemental Table 3). 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 1B and Supplemental Table 3). Although the increase in HR may mediate the observed change in %EF [28,29], 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 (Supplemental Figure 2).

We next examined gene expression in the ventricles of 3 month-old mice since such molecular changes frequently precede functional changes [30]. 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 1C).

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 1A) and significant hypertrophy was observed in males as compared to WT littermates (Figure 1B). Further, expression of fetal genes was increased and myosin expression was shifted toward a pathological state (elevated β*MyHC*) at this time-point (Figure 1D,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.

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 2A). 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 2A).

3.2 Prolonged α*MyHC-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 2B). Further we observed a trending increase in expression of a marker of activated, infiltrating macrophages, *Cd68* (Figure 2B). These pro-inflammatory, pro-fibrotic gene expression changes correlated with modest increases (2-fold) in left-ventricular fibrosis (Figure 2C) similar to that reported in models of hypertrophic cardiomyopathy and trans-aortic banding [31,32]. A trending increase in inflammatory cell presence was observed in α*MyHC-Cre*$^{+/-}$ ventricular

myocardium (Supplemental Figure 3). Comparable changes in gene expression were also confirmed in 6 month-old male αMyHC-Cre$^{+/-}$ mice (Supplemental Figure 4A). These pro-fibrotic gene expression changes may be directly attributable to activated p38 MAPK signaling (reviewed in [33]).

3.3 Prolonged α*MyHC-Cre* expression is associated with DNA damage response

Because Cre is a DNA recombinase, we hypothesized that the observed cardiotoxicity in αMyHC-Cre$^{+/-}$ mice may be attributable to Cre-related DNA damage. To this end, we examined DNA damage response effectors in female αMyHC-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 3A). These changes were accompanied by detectable transcriptional upregulation of master DNA damage response effectors, p21 and p53 in both female and male αMyHC-Cre$^{+/-}$ mice (Figure 3B, Supplemental Figure 4B) as well as a ~3-fold increase in cells with DNA fragmentation as identified by TUNEL-positive cells (Figure 3C).

3.4 Endogenous, degenerate *loxP* sites may be targeted by Cre following persistent expression under the α*MyHC* promoter

Given the absence of canonical *loxP* sites in the αMyHC-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, 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 [6,34] (Figure 4). Several hundred of these sites were identified, depending on the number of mismatches to the canonical site tolerated (Supplemental Table 4). 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 [35]. We next tested whether expression of 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 (Supplemental Figure 5A) and confirmed a change in protein expression consistent with the observed change in transcript expression for one target, *ME3* (Supplemental Figure 5B).

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) [25,26] to map the insertion site of the α*MyHC-Cre* transgene (Supplemental Figure 6A). 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 (Supplemental Figure 6B). We then used qPCR of gDNA in conjunction with standard curve generation to determine copy number of the α*MyHC-Cre*

transgene [24]. 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 (Supplemental Figure 7). Taken together, we do not believe insertion site or high copy number of the α*MyHC-Cre* transgene can account for the progressive cardiotoxicity observed in these mice.

5. 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 α*MyHC-Cre* mouse line [17–19]. 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 [13–15].

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 a primary insult [36–38]. 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), 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 isoform of Cre did not activate this pathway [9].

Although we did not observe gross changes in gene expression at many of the genes containing putative *loxP* sites (Supplemental Figure 5A), 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). 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*, Supplemental Figure 5A). 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 [39–42]. 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 [43]. Similar results were observed for α*MyHC-MerCreMer* mice in which animals on a mixed background (129Ola/C57Bl6/J) displayed less fibrosis than pure C57Bl6/J mice [13].

Sexually dimorphic phenotypes were observed at both timepoints examined, both by echocardiography and morphometric analysis (Figure 1 and Supplemental Table 3). Our subsequent analyses focused on female mice, given their demonstrated resistance to cardiac insult compared to males [44,45]. 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 (Supplemental Figure 4). 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 [46] in which myocardial Cre protein expression is considerably lower than the more commonly used, "conventional" α*MyHC* mice [1], cardiotoxicity was not observed in age-matched mice (Supplemental Figure 8). 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 [47] 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 α*MyHC-MerCreMer* model [13]. 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 [48]). Finally, we strongly encourage the use and separation of both sexes as molecular and phenotypic differences are common and clinically relevant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

αMyHC	alpha-myosin heavy chain
LoxP	locus of crossover P1
LADMA	Levenshtein Automata based Degenerate Motif Annotator
NFA	non-determinisitc finite state automata
PBS	Phosphate Buffered Saline
%EF	Percent Ejection Fraction
HR	Heart Rate
Anp	Atrial Naturietic Peptide
Bnp	Brain Naturietic Peptide
p38 MAPK	p38 Mitogen Activated Kinase
ERK	Extracellular-Signal-Regulated Kinase
PARP	poly (ADP-ribose) polymerase
Bax	BCL2-associated X protein
Ctgf	Connective tissue growth factor

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Highlights

- Widely used αMyHC-Cre transgenic mice display age-dependent cardiotoxicity.
- Cardiotoxicity is associated with fibrosis, inflammation, and DNA damage response.
- A novel platform was used to find degenerate *loxP* sites in the mouse genome.
- The mouse genome has hundreds of degenerate *loxP* sites that may be targeted by Cre.
- Off-target Cre effects at degenerate sites may contribute to cardiotoxicity.

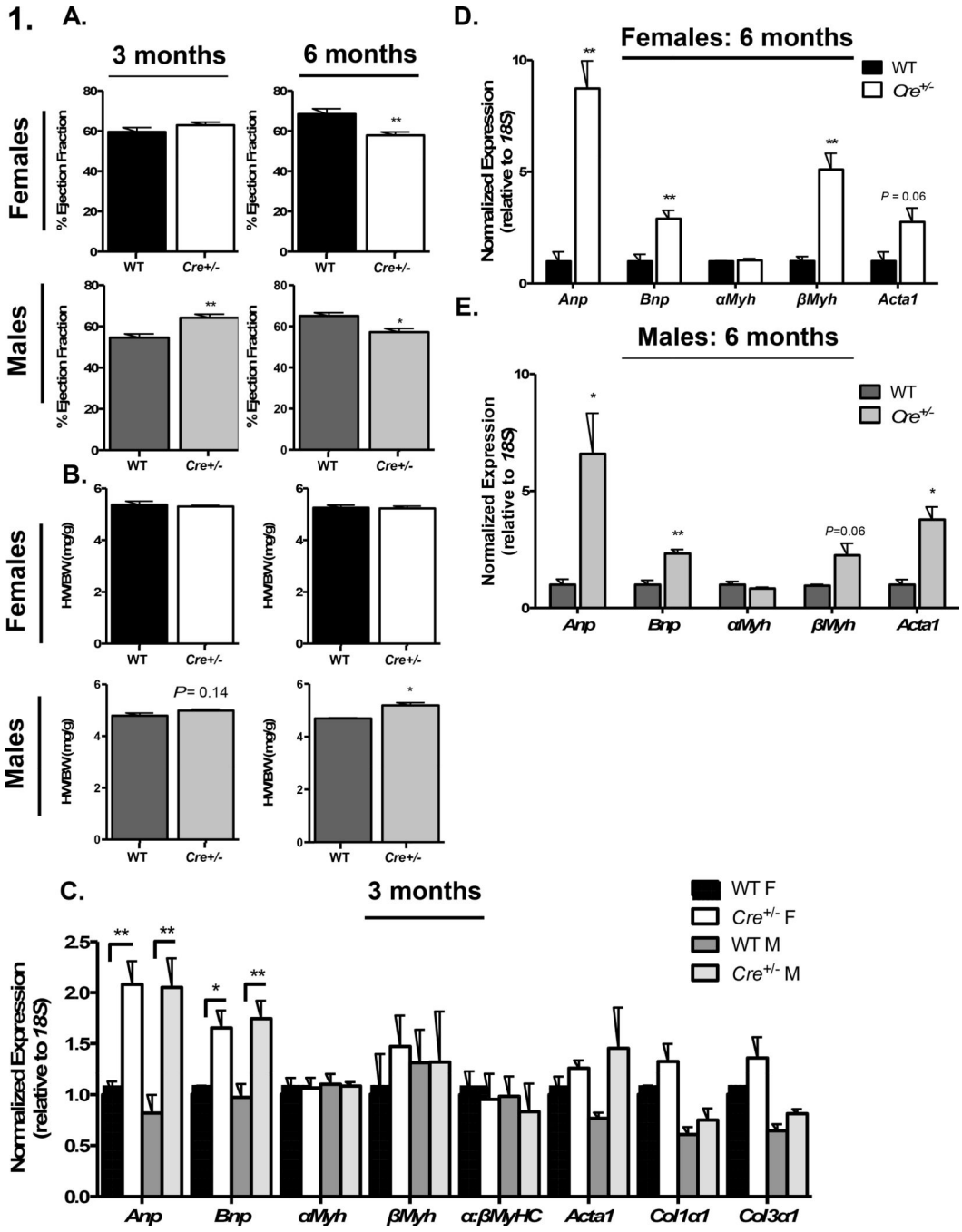

Figure 1. α*MyHC-Cre* is cardiotoxic by six months of age

(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.

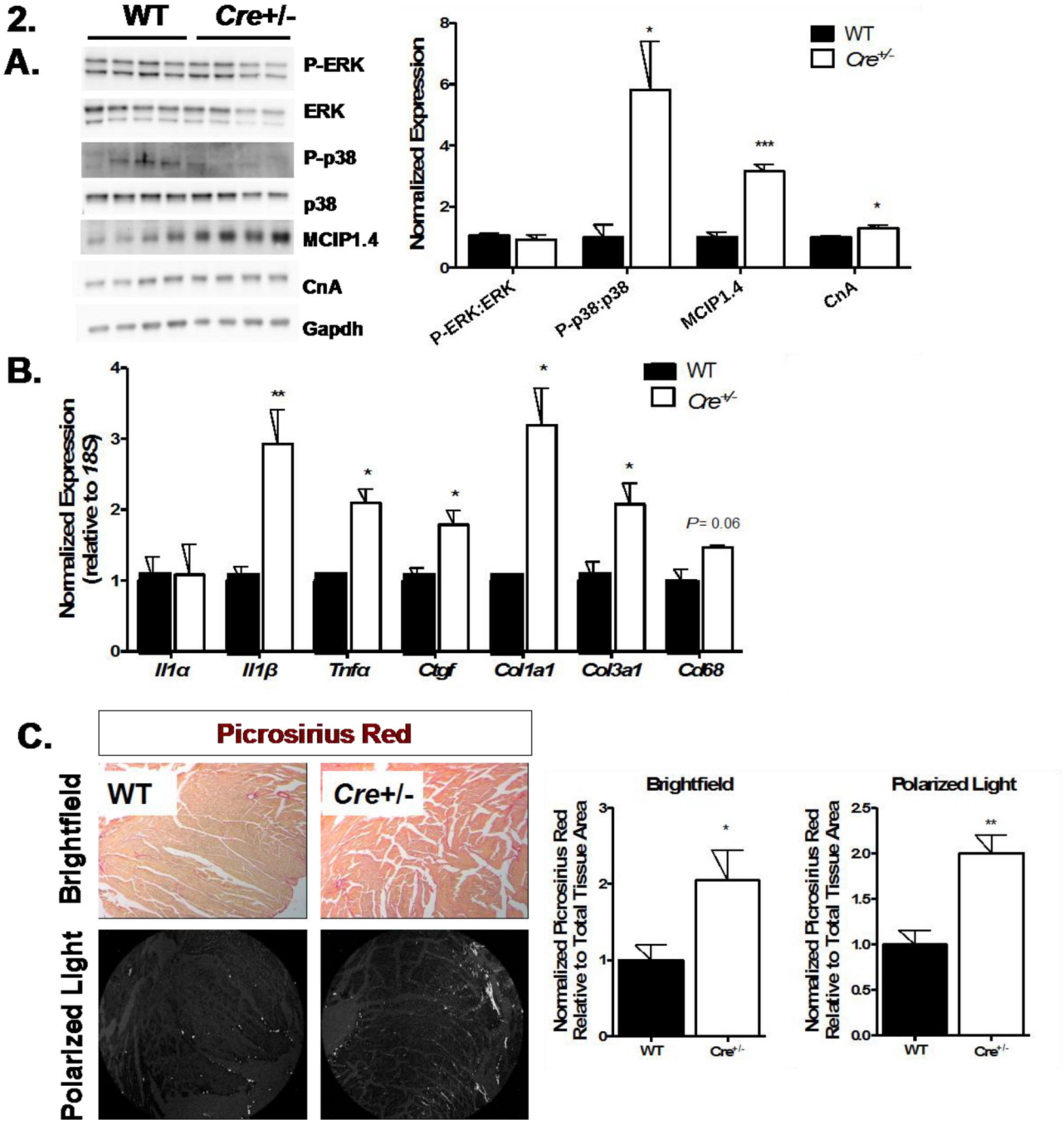

Figure 2. Induction of pathological intracellular signaling and inflammatory or fibrotic genes in female αMyHC $Cre^{+/-}$ ventricles at 6 months

(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) 10× 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.

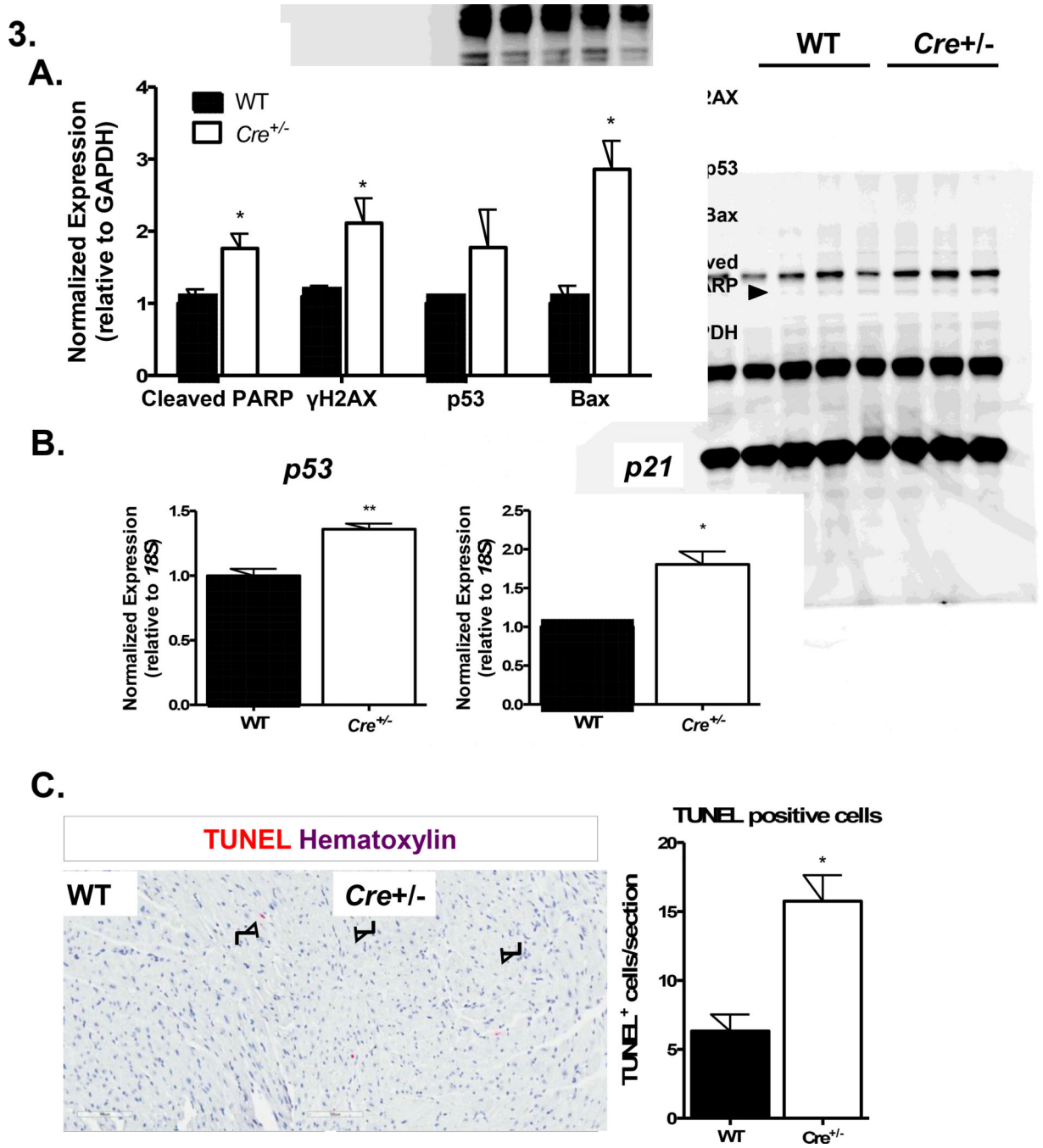

Figure 3. Ventricular DNA damage response in 6-month-old female αMyHC-Cre$^{+/-}$ mice

(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.

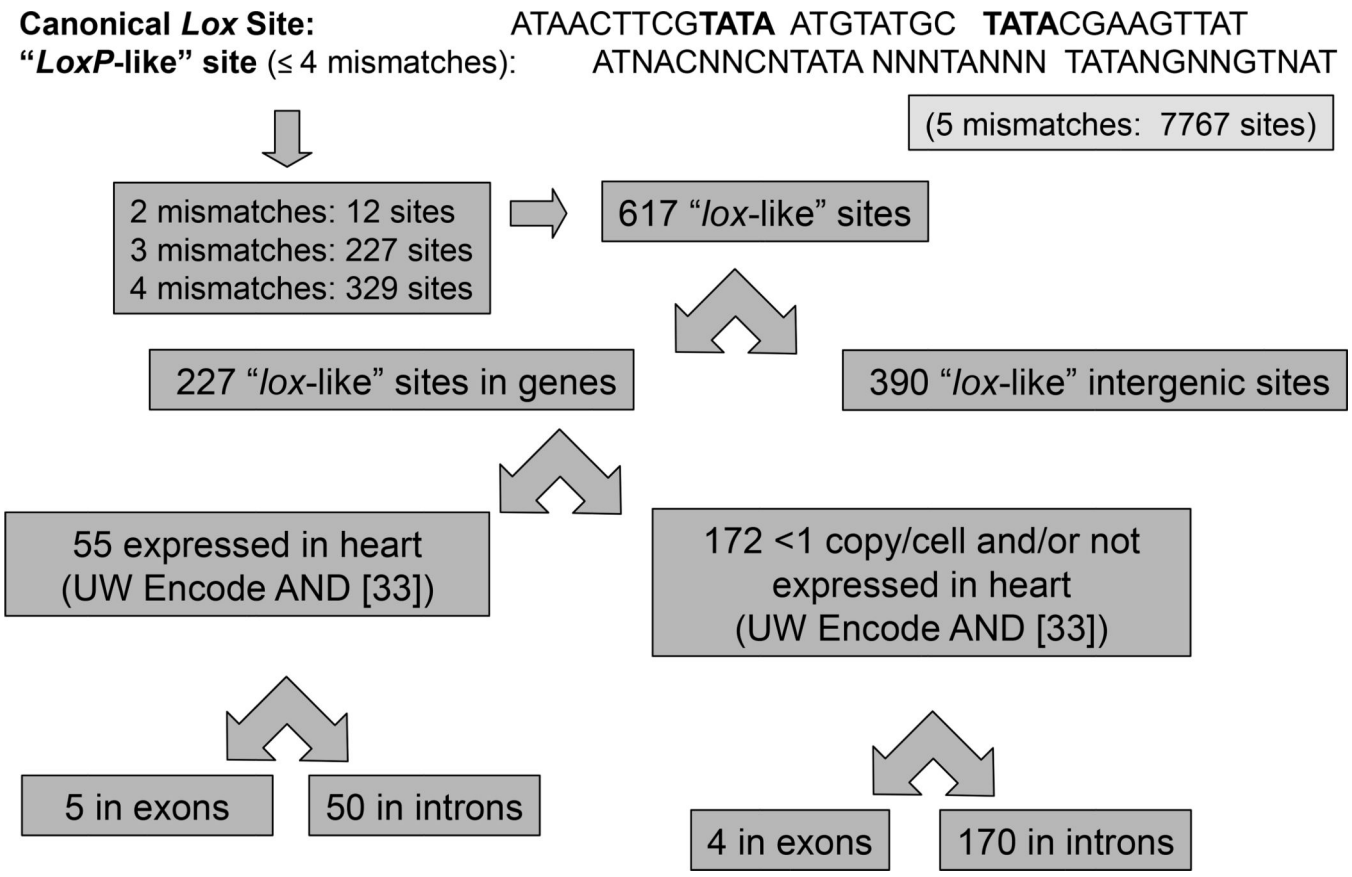

Figure 4. Degenerate *loxP* sites are abundant in the mouse genome

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 [6,34]. 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 [35].