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Cardiac-specific disruption of Bin1 in mice enables a model of stress- and age-associated dilated cardiomyopathy†

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Running Title: Bin1 deficiency causes dilated cardiomyopathy

Key Words: BAR domain, BAR adapter, Caveolin, $Ca_v1.2$, Dihydropyridine receptor (DHPR), lipid rafts, cardiomyocyte

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†This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1002/jcb.25198]

Additional Supporting Information may be found in the online version of this article.

Received 18 November 2014; Revised 13 April 2015; Accepted 14 April 2015
Journal of Cellular Biochemistry
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DOI 10.1002/jcb.25198

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Abbreviations

BAR, Bin/Amphiphysin/Rvs domain

DCM, dilated cardiomyopathy

eNOS, endothelial-type nitric oxide synthase

LV, left ventricle

LVID, left ventricle inner diameter

TAC, transverse aortic constriction

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Abstract

Non-compensated dilated cardiomyopathy (DCM) leading to death from heart failure is rising rapidly in developed countries due to aging demographics, and there is a need for informative preclinical models to guide the development of effective therapeutic strategies to prevent or delay disease onset. In this study, we describe a novel model of heart failure based on cardiac-specific deletion of the prototypical mammalian BAR adapter-encoding gene *Bin1*, a modifier of age-associated disease. *Bin1* deletion during embryonic development causes hypertrophic cardiomyopathy and neonatal lethality, but there is little information on how *Bin1* affects cardiac function in adult animals. Here we report that cardiomyocyte-specific loss of *Bin1* causes age-associated dilated cardiomyopathy (DCM) beginning by 8-10 months of age. Echocardiographic analysis showed that *Bin1* loss caused a 45% reduction in ejection fraction during aging. Younger animals rapidly developed DCM if cardiac pressure overload was created by transverse aortic constriction. Heterozygotes exhibited an intermediate phenotype indicating *Bin1* is haplo-insufficient to sustain normal heart function. *Bin1* loss increased left ventricle (LV) volume and diameter during aging, but it did not alter LV volume or diameter in hearts from heterozygous mice nor did it affect LV mass. *Bin1* loss increased interstitial fibrosis and mislocalization of the voltage-dependent calcium channel $Ca_v1.2$, and the lipid raft scaffold protein caveolin-3, which normally complexes with Bin1 and $Ca_v1.2$ in cardiomyocyte membranes. Our findings show how cardiac deficiency in Bin1 function causes age- and stress-associated heart failure, and they establish a new preclinical model of this terminal cardiac disease. This article is protected by copyright. All rights reserved

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Introduction

Non-compensated dilated cardiomyopathy (DCM), which eventually leads to death from heart failure, is increasing in incidence in developed countries due to aging demographics. Effective methods to delay disease onset either at the level of normal function or compensated DCM would be of great clinical value. However, epigenetic and genetic factors that may modify susceptibilities and direct treatments are poorly understood as yet, particularly in the case of sporadic disease. Aging and hypertension are major risk factors in disease development, therefore, studies demonstrating age-associated or pressure-associated disease development would permit the evaluation of mechanism-based therapeutic approaches. While genetically defined models of DCM that are modified by aging and pressure changes in the heart have been identified (e.g. (Mende et al. 2001, Son et al. 2007, Swinnen et al. 2009, Wang et al. 2009)), there nevertheless remains insufficient knowledge of the mechanisms involved in DCM, where relevant preclinical models are needed to explore prognostic tools and therapeutic options.

BAR adapter proteins are pleiotropic regulators of membrane dynamics and nuclear functions, with roles in endocytosis, vesicle fusion and trafficking, specialized membrane organization, actin organization, cell polarity, stress signaling, transcription, immune modulation and tumor suppression (Ren et al. 2006, Prendergast et al. 2009). This family of adapter proteins originally identified through discovery of *Bin1* (Sakamuro et al. 1996) is now recognized as a subset of a larger superfamily of structurally-related proteins that includes the F-BAR and I-BAR proteins (Casal et al. 2006, Dawson et al. 2006, Itoh and De Camilli 2006, Chitu and Stanley 2007). The BAR domain is the signature domain for which this protein superfamily is named. BAR domains mediate oligomerization to form a banana-shaped dimer that interacts with curved membranes,

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small GTPases and other membrane bound, cytosolic, and nuclear proteins (Habermann 2004, Peter et al. 2004, Ren et al. 2006). While its full functionality is complex (Prendergast et al. 2009) biochemical and structural studies have defined shared canonical functions for BAR domains in bending and tubulating membranes, and in facilitating interactions with the actin cytoskeleton (Peter et al. 2004, Itoh and De Camilli 2006).

As one of the founding members of the BAR protein superfamily, *Bin1* encodes the prototypical eukaryotic amphiphysin that is broadly expressed and conserved throughout evolution from yeast to human (Prendergast et al. 2009). Beyond its functions in vesicle trafficking and cytoskeletal actin organization, Bin1 exerts specific functions in cell polarity, immune control and stress signaling, including in the nucleus where certain splice isoforms localize along with other endocytic proteins (Prendergast et al. 2009, Pyrzynska et al. 2009). Notably, genetic studies in mice have discriminated non-essential roles in endocytic processes commonly ascribed to amphiphysins from the essential roles of *Bin1* in the growth, survival and motility of stressed cells (Prendergast et al. 2009). In particular, *Bin1* has been implicated in cancer suppression, acting in default pathways of senescence, apoptosis and immune surveillance licensed by activation of the Raf or Myc oncogenes (DuHadaway et al. 2001, Muller et al. 2005, Wajapeyee et al. 2008). Overall, genetic investigations in yeast, fruit flies and mice suggest that Bin1 integrates polarity-associated stress signals to maintain proper membrane dynamics, thereby modifying the action of multiple pathways of cell proliferation, survival, motility and immune control (Prendergast et al. 2009).

Targeted deletion of *Bin1* in mice resulted in perinatal lethality associated with onset of a severe hypertrophic cardiomyopathy (Muller et al. 2003) indicating a critical developmental role for Bin1 in the heart. Clinically, low Bin1 levels in plasma have recently been associated with heart failure (Hong et al. 2012). *Bin1* is highly expressed in muscle, where *Bin1* functions in differentiation and

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T tubule formation (Wechsler-Reya et al. 1998, Lee et al. 2002). Related functions in skeletal and cardiac muscle are suggested by evidence that Bin1 co-localizes in both tissue types with the lipid raft protein caveolin-3 (Cav-3) and the L-type calcium channel 1.2 ($Ca_v1.2$), which is the target of the calcium channel blocking drug dihydropyridine used for the treatment of hypertension (Lee et al. 2002, Nicot et al. 2007). Further, in both mouse and human adult cardiomyocytes Bin1 interacts with $Ca_v1.2$ and this interaction is critical for its calcium signaling function at T tubules (Hong et al. 2012). Taken together, these observations prompt the hypothesis that Bin1 is a positive modifier of cardiac contractility that helps sustain adult heart function under stress conditions. In this study, we offer direct genetic evidence in support of this hypothesis, using a conditional mouse model in which *Bin1* is deleted specifically in cardiomyocytes.

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Materials and Methods

In vivo generation of the cardiac-specific Bin1KO mouse model (CaKO). Because of the perinatal lethality of homozygotic Bin1 null animals, we mated animals carrying a Bin1 flox allele with mice carrying one copy of the constitutive Bin1 null allele and a cardiac-specific Cre transgene. We bred homozygotic $Bin1^{flox/flox}$ mice (loxP sites around exon 3 of the *Bin1* gene) with $Bin1^{+/-}$:αMHC-$Cre^{+/-}$ transgenic mice to drive loxP recombination in the heart (Agah et al. 1997) The transgenic $Bin1^{flox/flox}$ mice and animals carrying the constitutive null Bin1 allele ($Bin1^{+/-}$) have been described previously (Muller et al. 2003, Chang et al. 2007). The resulting genotypes of the progeny were confirmed by PCR to differentiate between progeny carrying the floxed, null and Cre alleles (Muller et al. 2003, Chang et al. 2007). Littermates were used in the studies and have the following genotypes: control wildtype mice (+/flox) are $Bin1^{+/flox}$:αMHC-$Cre^{-/-}$, heterozygotic mice (+/Δ) are $Bin1^{+/flox}$:αMHC-$Cre^{+/-}$, and cardiac-specific Bin1 null mice (-/Δ) are $Bin1^{-/flox}$:αMHC-$Cre^{+/-}$. Animal work was performed with Institutional Animal Use and Care Committee approval.

In vivo functional cardiac analysis by echocardiography. Echocardiography was done using the VisualSonics Vevo 770 High-Resolution Imaging System with scan-head 707B for mouse cardiac imaging (focal length 12.7 mm). Animals were monitored for body temperature, respiration rate and heart rate. The parasternal long axis view in B or M mode was used to measure left ventricular outflow tract, stroke volume, cardiac output, intraventricular septum thickness, left ventricular inner diameter, and left ventricular posterior wall thickness at diastole and systole. A parasternal short axis view was obtained to confirm M-mode measurements on the center of the left ventricle. Measurements of flow through the mitral valve and tricuspid valve were done on the apical four chamber view using pulse wave doppler. Pulse wave doppler of both the ascending and descending

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areas of the aorta, in aortic arch view, provided blood flow measurements, aortic velocity time integral, and atrioventricular peak velocity. Measurements from the suprasternal view determined aortic valve function and confirmed aortic arch view measurements.

Transverse aortic constriction (TAC). In vivo pressure overload was induced surgically by banding the transverse aorta, as described previously (Akhter et al. 1998, Manning et al. 2000). Sham-operated animals underwent the same surgical procedure without banding the transverse aorta. Operative mortality was below 50% in all groups.

Western blot analysis. Cell and tissue protein lysates were prepared in RIPA buffer containing protease and phosphatase inhibitors (Huang et al. 2007). Equal protein for each sample (typically 50-100 µg/lane) was separated by SDS–PAGE and blotted to Immobilon-P transfer membrane (Millipore, Billerica MA). Blots were incubated with primary antibodies as recommended by the vendor and were detected with HRP conjugated secondary antibodies using the ECL reagents according to the manufacturer's instructions and autoradiography. Scans of autoradiographic films was adjusted to gray scale. Primary antibodies to the following antigens were used: Cav3 (BD Transduction Labs, San Diego CA); $Ca_v1.2$ (Alomone Acc-003, Jerusalem Israel).

Lipid raft analysis. Lipid raft/caveolae-enriched membrane fractions were purified as described previously (Schubert et al. 2002). Hearts were homogenized in Mes-buffered saline (25 mM Mes, pH 6.5, 0.15 M NaCl) containing protease and phosphatase inhibitors (Sigma, St. Louis MO). Homogenization was carried out using a Polytron tissue grinder. Triton X-100 was added to the homogenate to achieve a final concentration of 1% and incubated on ice for 30 minutes. After low-speed centrifugation, the homogenate was adjusted to 40% sucrose by the addition of 1 ml of 80%

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sucrose prepared in Mes-buffered saline, transferred to an ultracentrifuge tube, and overlaid with a discontinuous sucrose gradient of 30% sucrose and of 5% sucrose (also in MBS buffer without Triton X-100), respectively. The samples were centrifuged at 39,000 rpm for 16-20 h in a SW41 rotor (Beckman Instruments, Palo Alto, CA). Twelve 1 ml fractions were collected, and equal aliquots of each fraction were subjected to SDS−PAGE and immunoblotting.

Histology. Tissue was fixed in 4% paraformaldehyde and paraffin embedded. Sections were either stained with Hematoxylin and Eosin, Trichrome or Oregon green conjugated wheat germ agglutin (Molecular Probes, OR) to determine the extent of fibrosis. To visualize nuclei, cells were stained with 4',6-diamidino-2-phenylindole (DAPI) in the mounting medium, before examination by fluorescence microscopy. Fluorescent signal was analyzed using a Zeiss Axiovert 220 microscope powered by Axiovision 4.0 software with multi-channel/Z-stack acquisition and 3D-deconvolution modules.

Statistical analysis. The student's t-test was used to compare the difference between groups at various ages or after TAC. The differences were considered to be statistically significant at p-values less than or equal to 0.05.

Results

Cardiomyocyte-specific deletion of *Bin1* in mouse limits cardiac function during aging. Previous work in mice revealed that Bin1 is necessary for cardiac function, based on findings that *Bin1* null neonates died within 24 hours after birth presenting with severe ventricular hypertrophy (Muller et al. 2003). To determine whether Bin1 deficiency would directly affect heart function after birth, we generated three mouse strains carrying a conditional flox allele, the null allele and a

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cardiac-specific, αMHC promoter-driven Cre transgene. A schematic of the breeding is presented along with results confirming the in vivo rearrangement of the 'floxed' allele in the heart of 1 day old neonates (Figure 1). The mice expressing (Bin1+/flox) or deficient in *Bin1* (Bin1 +/Δ and Bin1 -/Δ) developed normally. Echocardiography was begun at 2 months of age and continued until 15 months of age. Subsequently, Bin1 -/Δ mice died at ~16 months of age, prematurely without a gender bias. Cardiac parameters obtained from Bin1+/flox, Bin1 +/Δ and Bin1 -/Δ animals were depicted graphically over time (Figure 2). By 12 months of age there was a significant relative decrease in Bin1 -/Δ mice in the percent ejection fraction (-45%) and the percent fractional shortening (-48%), relative to Bin1 +/flox mice which retained full gene function. Similarly, in heterozygous Bin1 +/Δ mice at 12 months of age, while there was a statistically significant decrease in both the percent rejection fraction (-27%) and the percent fractional shortening (-31%), these reductions in cardiac output did not continue to trend downward in older mice of 15 months of age, but instead lessened.

Notably, cardiac-specific loss of *Bin1* significantly affected left ventricular (LV) diameter and volume at a relatively early age, as compared to Bin1+/flox mice which retained full gene function (Figure 2). *Bin1* loss accounted for a 10% change in the LV inner diameter at 3 months of age and ~20% at 12 through 15 months of age. LV volume also increased with a 26% difference at 3 months of age and >50% at 12 through 15 months of age. A slight increase in LV volume was noted in heterozygous Bin1 +/Δ mice at 3 months of age, however, there was not a statistically significant difference in LV volume at older ages despite some apparent trend. Cardiac-specific loss of *Bin1* also significantly altered cardiac output as animals aged, with a 29% decrease in output by 15 months of age, when compared to Bin1+/flox mice. LV mass did not significantly change within all the strains tested or between them (Figure 2). Representative echocardiographic images are

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presented for each genotype in Supplemental Figure 1. To rule out the possibility that Cre-mediated toxicity might contribute to cardiac decline in the Bin1-deficient mice, as reported in other models (Schmidt-Supprian and Rajewsky 2007, Takimoto et al. 2009), we also measured cardiac function in Bin1 +/+; Cre+/- mice. We observed no loss in cardiac function by 12 months of age when compared to the fully functional Bin1 +/flox mice that did not express Cre recombinase (data not shown). Thus, Cre expression did not significantly alter heart function in this model. Together, our results indicated that *Bin1* loss in cardiomyocytes caused progressive development of dilated cardiomyopathy, as determined by lower ejection fractions, fractional shortening and increased LV volume.

Bin1 deficient hearts display increased interstitial fibrosis and mislocalization of caveolin-3 and $Ca_v1.2$ from lipid rafts. To explore the phenotypic consequences of a cardiac deficiency in Bin1, we evaluated the extent of fibrosis in the hearts from Bin1+/flox, Bin1 +/Δ and Bin1 -/Δ mice. At 3 months of age, we observed little difference in fibrous content in these mice (data not shown). However, by 15 months of age, moderate fibrosis was observed in hearts from Bin1 +/Δ that lost one allele mice and extensive fibrosis was seen in hearts from Bin1 -/Δ mice that lost both alleles (Figure 3).

Bin1 has been reported previously to associate with caveolin-3 and $Ca_v1.2$ which support cardiac function (Lee et al. 2002, Nicot et al. 2007). Therefore, we examined the levels of expression of these proteins in cardiomyocytes from the mice at young and old ages. Little to no change was observed in caveolin-3 or $Ca_v1.2$ levels in hearts from young (2 month) or old (15 month) mice of any genotype (Supplemental Figure 2). The BAR domain of Bin1 enables membrane interaction, however, other domains in Bin1 are responsible for its localization to additional sites within cells.

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Because caveolin-3 is primarily associated with lipid rafts, we performed a Western analysis of fractionated tissues extracts to examine whether Bin1 loss affected the subcellular distribution of caveolin-3 and $Ca_v1.2$ in the heart (Figure 4). While aging generally influenced the distribution of both proteins in all the mice, Bin1 insufficiency produced a pronounced shift in the distribution of caveolin-3 and $Ca_v1.2$ to either the detergent-insoluble (fractions 4 & 5) or detergent-soluble (fractions 11 & 12) fractions, with reduced partitioning in intermediate fractions (fractions 6 through 10). To confirm these results, we also compared the intracellular localization of caveolin-3 and $Ca_v1.2$ by immunostaining of fixed heart tissues from +/flox, +/Δ and –/Δ mice (Figure 5). Consistent with the lipid raft fractionation data, there was a partial loss of the characteristic banding pattern seen in +/flox heart in the partially deficient +/Δ heart, and a complete loss of this pattern in fully deficient –/Δ heart. Overall, these observations offered potential insights into the observed disturbances in heart function caused by loss of Bin1 in cardiomyocytes.

Cardiac stress accelerates the loss of cardiac function in Bin1 deficient mice. The cardiac-specific loss of *Bin1* results in a decline in heart function over a period of 15 months. In order to determine whether cardiac stress would influence the extent of dysfunction observed in the Bin1 deficient mice, we utilized the Transverse Aortic Constriction model (TAC). At 2 months of age, mice either underwent the TAC banding surgery or a sham procedure without aortic banding. Echocardiography was done before and after surgery. Figure 6 shows the graphical analysis of the cardiac parameters obtained from Bin1+/flox, Bin1 +/Δ and Bin1 -/Δ animals. No changes in ejection fraction or fractional shortening were observed in animals that underwent the sham procedure, regardless of their Bin1 status (see Supplemental Figure 3). In contrast, by 8 months of age, *Bin1* deficiency led to a 39% reduction in ejection fraction in Bin1-/Δ mice and 44% in Bin1+/Δ mice, relative to control Bin1+/flox mice ($p<0.01$). The difference in ejection fractions

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between the Bin1 -/Δ and +/Δ groups was not statistically significant (p=0.703). Similarly, Bin1 loss resulted in a 43% change in fractional shortening in Bin1-/Δ mice and 49% in Bin1+/Δ mice, relative to control Bin1+/flox mice ($p<0.01$). As before, the difference in fractional shortening between the Bin1 -/Δ and +/Δ groups was not significant (p=0.700). By 8 month after TAC surgery, hearts with insufficient Bin1 expression also demonstrated changes in LV volume and inner diameter during systole, but not diastole. When compared to Bin1 +/flox controls, there were respective 24% and 71% increases in LVID and LV volume in Bin1 -/Δ mice, and respective 32% and 100% increase in LVID and LV volume in Bin1 +/Δ mice. These parameters were significantly different from controls ($p<0.05$). While no significant differences were observed in the changes of LV volume and LVID (left ventricle inner diameter) during diastole, or in LV mass, we observed a 26% reduction in cardiac output in the Bin1-/Δ group, which was significantly different from the control group (p=0.02). Overall, given the similar phenotypes of nullizygous and heterozygous mice, it was clear that wild-type levels of Bin1 expression were necessary to adapt to cardiac stresses, such as pressure overload, suggesting that *Bin1* is haplo-insufficient to preserve heart function in animals under stress.

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Discussion

The primary finding of our study is that cardiac-specific attenuation of the non-sarcomeric protein Bin1 results in heart failure. This study considerably extends and deepens earlier findings that Bin1 is essential for normal heart development (Muller et al. 2003) by revealing the continued importance of Bin1 in the adult heart. Aging-related changes occur in the heart and our results suggest that Bin1 is an integral protein needed for maintaining normal cardiac function and that its levels alter heart function with increasing age. Additionally, using a model of left ventricular pressure overload (TAC), we found that Bin1 expression also acts to preserve cardiac function under stress. Inducing pressure overload not only accelerated the onset of LV dysfunction in the hearts of mice with Bin1 cardiomyocyte deficiency, but it also diminished function in mice lacking just one *Bin1* allele, suggesting that adequate Bin1 levels are needed to sustain heart function under pressure stresses. These findings genetically validate the clinical observation that cardiomyocytes from failing human hearts exhibited decreased BIN1 expression levels (Hong et al. 2012). It has also been reported that diminished Bin1 levels in failing human hearts alter the localization of the L-type calcium channel protein Ca_v 1.2 and impair calcium release (Hong et al. 2012), and lower plasma Bin1 levels were found to be associated with heart failure (Hong et al. 2012). Consistent with the possibility that Bin1 status may be a crucial determinant of heart function, a whole genome analysis of a German family exhibiting autosomal dominant cardiomyopathy defined human chromosome 2q14-q22 where the BIN1 gene is located as the most likely disease locus (Negorev et al. 1996, Jung et al. 1999) Taken together, our findings add to a growing body of work strongly suggesting that reduction of Bin1 expression negatively affects heart function, leading to DCM and heart failure.

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One important implication of our work is the idea that Bin1 is a modifier of heart function during aging. This implication extends earlier work suggesting Bin1 as a generalized modifier of age-associated disease, for example, in the setting of cancer where Bin1 exerts tumor suppressor activities manifested during aging or after the initiation of oncogenic stress (Chang et al. 2007, Chang et al. 2007, Prendergast et al. 2009), or in the setting of Alzheimer's disease where Bin1 has been defined as a major susceptibility locus for the most common late-onset form of this dementia (Hollingworth et al. 2010, Jones et al. 2010, Seshadri et al. 2010, Belbin et al. 2011). Herein, we suggest that in the heart Bin1 serves as a positive modifier of cardiac function during aging and cardiac stress, based on our findings of the important role that normal Bin1 expression plays to protect the heart. Bin1 functionality is complex and the same mechanisms involved in suppressing cancer may be distinct from those suppressing heart failure during aging. For example, while Bin1 was identified originally by the suppressive effects of certain alternate splice isoforms on the Myc oncoprotein, we did not observe Myc activation in Bin1-deficient hearts (unpublished observations) despite other evidence that Myc influences heart function (Moens et al. 1993). On the basis of our work to date, the action of Bin1 in cardiac myocytes is less likely to involve Myc pathways than other interactions such as the Caveolin-3 and $Ca_v1.2$ pathways discussed below.

Extending studies in striated muscle (Lee et al. 2002, Muller et al. 2005, Hong et al. 2012), we found that Bin1 loss altered the biochemical and lipid raft distribution of Caveolin-3 and $Ca_v1.2$ in cardiomyoctes and that normal localization patterns changed with age. It is likely that the cumulative effects of losing Caveolin-3 and $Ca_v1.2$ at T tubules during aging would cause inherent stresses that promote heart failure, given the importance of the L-type calcium channel to cardiomyocyte contractility which cannot be overstated. For example, alterations in calcium currents through modulation of $Ca_v1.2$-associated subunit β2a lead to pathological cardiac function

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(Nakayama et al. 2007, Chen et al. 2011). Overall, it is clear that complex channel formation and membrane localization events are critical determinants of T-tubule integrity that is vital for cardiac function (Bers 2002, Bodi et al. 2005, Jaleel et al. 2008).

T-tubules develop progressively after birth and are necessary for synchronized calcium-induced calcium release in myocytes (Orchard et al. 2009, Louch et al. 2010). These dynamic structures are remodeled during the development of heart failure (Louch et al. 2010, Wei et al. 2010). After TAC surgery, T-tubule remodeling occurs during the compensation phase of hypertrophy before LV dysfunction is observable by echocardiography (Wei et al. 2010). The significance of appropriate $Ca_v1.2$ subcellular localization to T-tubule function is documented along with the connection of Bin1 to this vital process. Hong and coworkers demonstrated direct Bin1 and $Ca_v1.2$ interactions and established that Bin1 is critical to anchor $Ca_v1.2$ to T-tubular membranes, based on evidence that loss of Bin1 expression altered subcellular localization of $Ca_v1.2$, thus altering $Ca_v1.2$ expression and calcium and potassium currents (Hong et al. 2012, Hong et al. 2014). Moreover, a recent study published after submission of this paper using our same Bin1 cardiac-specific 'flox' model obtained direct evidence of functional significance to the caveolin-3 and $Ca_v1.2$ alterations caused by Bin1 loss, through a gene rescue experiment in a non-cardiac cell type (Hong et al. 2014). In summary, our findings strengthen and extend the biochemical and functional significance of direct Bin1 and $Ca_v1.2$ interactions in the setting of the aging and stressed heart as it relates to the development of DCM and heart failure.

Loss of Caveolin-3 in the heart similarly diminishes heart function and leads to cardiomyopathy (Woodman et al. 2002, Gazzerro et al. 2010). As with cardiac-specific loss of Bin1, signs of diminished cardiac function appear after 15 months of age in caveolin-3 knockout mice. Caveolin-3

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loss was reported to alter ERK signaling, but we did not see this alteration with Bin1 loss (unpublished observations). Interestingly, human mutations in Caveolin-3 appear to primarily affect skeletal muscle, as is the case with Bin1 (Nicot et al. 2007, Fernando et al. 2009, Gazzerro et al. 2010, Toussaint et al. 2010) Significant work has emerged describing the importance of Bin1 in skeletal muscle. Centronuclear myopathy of the skeletal muscle results from mutations in the BIN1 locus or mutations in proteins identified as interacters or modifiers of Bin1 (Nicot et al. 2007, Fernando et al. 2009, Gazzerro et al. 2010, Toussaint et al. 2010, Fugier et al. 2011, Bohm et al. 2013, Royer et al. 2013). Bin1 isoforms generated by alternate splicing are specifically expressed in skeletal muscle, where a unique phosphotidylinositol binding motif is included only in this tissue (Wechsler-Reya et al. 1998, Lee et al. 2002). Insofar as all Bin1 isoforms were ablated in our model, future investigations might focus on these aspects to pursue theranostic avenues of clinical relevance, including potential strategies to delay or reverse disease onset by influencing cardiac remodeling.

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Acknowledgements

The authors declare that they do not have any competing or financial interests. G.C.P. is an inventor on U.S. and international patents claiming human and murine Bin1 sequences and antibodies. This work was supported by the Delaware Affiliate of the American Heart Association (0855499D to LLK), the National Cancer Institute (CA100123 and CA82222 to GCP), the Lankenau Medical Center Foundation and the Main Line Health System.

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Figure Legends

Figure 1: **Rearrangement of the Floxed *Bin1* Allele Occurs in Mice Carrying α-MHC Promoter Driven Cre Recombinase**. *A: Bin1* +/-:αMHC/Cre +/- mice were crossed with *Bin1* flox/flox transgenic mice. Progeny numbered 1 through 3 were used in the experiments. Heart genomic DNA from the offspring was evaluated for Cre-mediated recombination by PCR. Both recombined (Δ, loxP 0.3kb) and intact (flox 1.1kb) alleles can be observed in the DNA and can be discerned from the *Bin1* wildtype (+, 0.9kb) allele. The molecular weight marker (M) is shown. B: Cre recombinase expression in the heart from one month old Bin1+/Δ and Bin1-/Δ αMHC/Cre transgenic mice.

Figure 2: Echocardiographic Analysis from Cardiac-specific Bin1 Expressing and Deficient Mice. Heart function was assessed as the mice aged by examining % Ejection Fraction, % Fractional shortening, LVID (left ventricular inner diameter) in diastole, LV Volume in diastole, LV mass and CO. Graphs for the functional parameters are presented indicating the parameter on the y-axis and months of age on the x-axis. Data (AVE±SEM) for Bin1 +/flox (n=5-11 measurements per age group), Bin1+/Δ (n=8-11 measurements per age group) and Bin1-/Δ (n=9-15 measurements per age group) are shown. * significant at p≤0.05, ** significant at p≤0.01, *** significant at p≤0.001. LV, Left Ventricle; LV Volume (unit, microliters); LV mass (unit, mg); LVID (d), Left Ventricle Inner Diameter at diastole (unit, mm); CO, cardiac output (unit, ml/min); %EF is the percent ejection fraction, which describes the percentage of blood leaving the heart during a contraction; it is based on the formula %EF=Stroke volume ejected from the ventricles at each heart beat / End diastolic volume x100. %FS is the percent change in diameter of the left

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ventricle; it is based on the formula %FS=LV end diastolic diameter – LV end systolic diameter) / LV end diastolic diameter x100.

Figure 3: Bin1 Deficient Hearts Exhibit Increased Interstitial Fibrosis by 15 months of age. Heart tissue sections from Bin1+/flox, Bin1+/Δ and Bin1-/Δ transgenic mice were stained with Hematoxylin/Eosin (left), labeled with wheat germ agglutinin-oregon green 488 and DAPI (middle), or stained with Trichrome (right). Serial sections were cut beginning at approximately 50 microns into the ventricles from the apex of the heart. These sections are at comparable depths within the tissue. The arrows highlight regions of fibrosis in the sections. Scale bar, 50 μm. Representative data is presented from minimally 3 mice examined per genotype.

Figure 4: Western Analysis of Lipid Raft Gradient Fractions. Triton X-100 insoluble proteins was extracted from hearts isolated from 2 or 15 month of animals that expressed or were deficient in Bin1. Western analysis was performed on the gradient fractions 3-12 to assess localization of caveolin-3 and $Ca_v1.2$. Representative data is presented from minimally 3 experiments.

Figure 5: Bin1 Deficient Hearts Exhibit Age-Associated Disorganization of T-tubular Caveolin 3 and $Ca_v1.2$. Serial sections were cut beginning at approximately 50 microns into the ventricles from the apex of the heart. These sections were at comparable depths within tissues. Immunofluorescence was performed to visualize caveolin-3 or $Ca_v1.2$ using appropriate secondary antibodies. DAPI staining was used to visualize nuclei. Scale bar, 20μm. Representative data is presented from minimally 3 mice examined per genotype.

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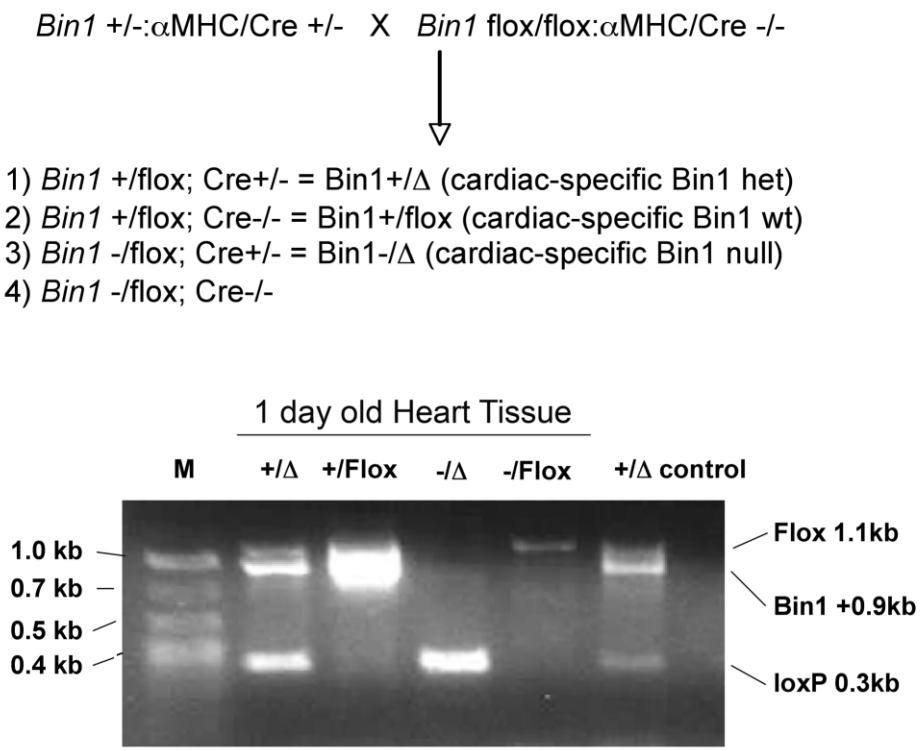
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Figure 6: Echocardiographic Analysis of Cardiac-specific Bin1 Expressing and Deficient Mice after TAC surgery. Heart function was assessed by examining % Ejection Fraction, % Fractional Shortening, LVID in systole and diastole, LV Volume in systole and diastole, LV mass and CO. Graphs for the functional parameters are presented indicating the parameter on the y-axis and time post surgery on the x-axis. Data (AVE±SEM) for Bin1+/flox (n=6-10 measurements per time point), Bin1+/Δ (n=5-6 measurements per time point) and Bin1–/Δ (n=6-7 measurements per time point) are shown. * significant at $p \leq 0.05$, ** significant at $p \leq 0.01$.

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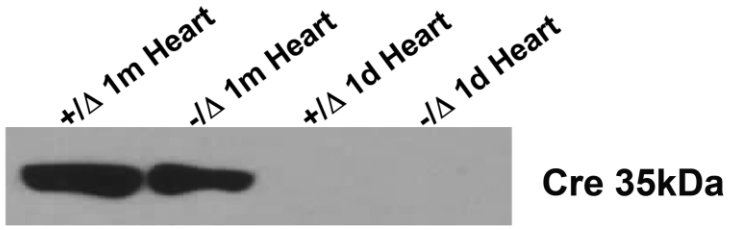


Figure 1

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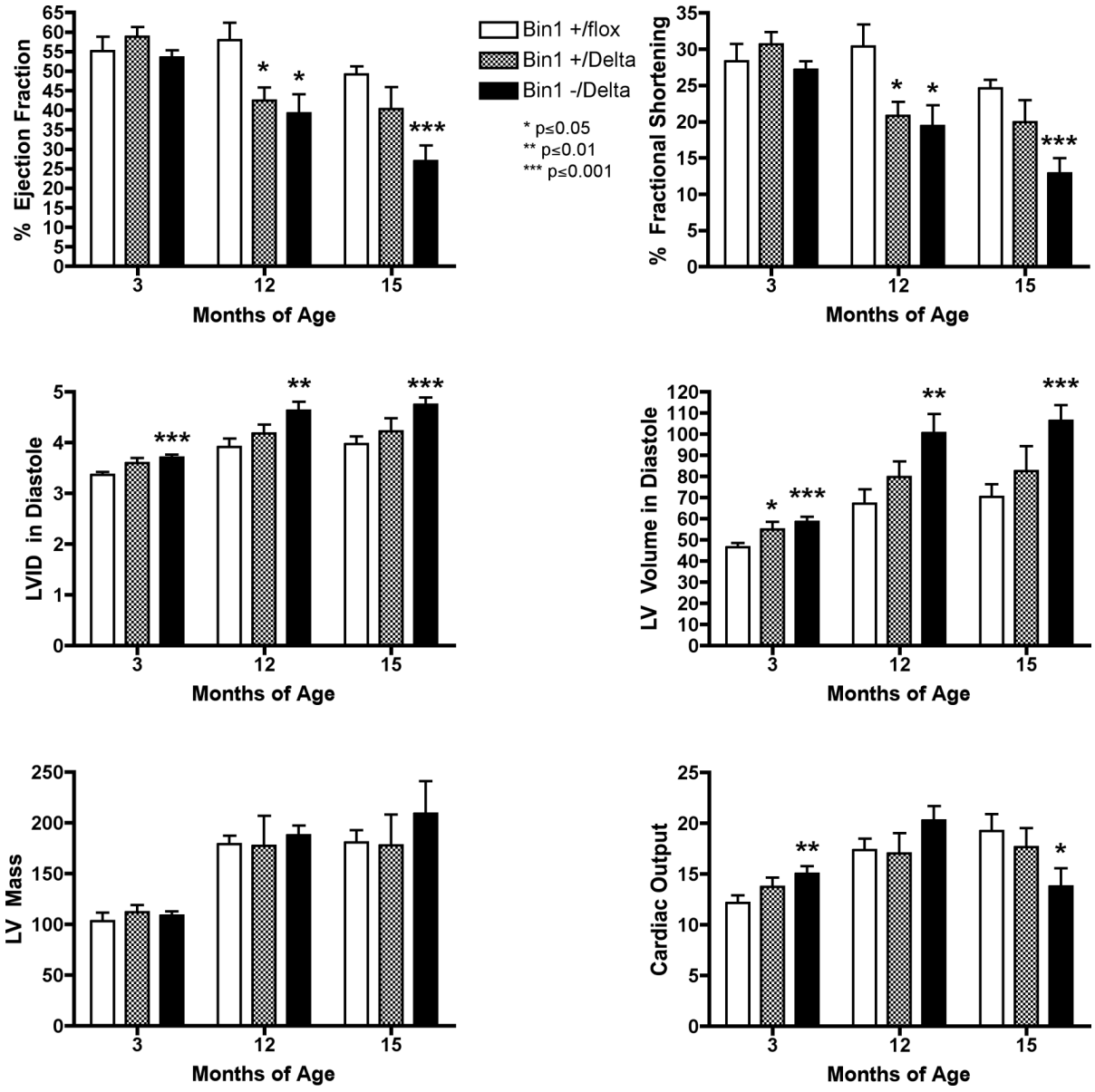


Figure 2

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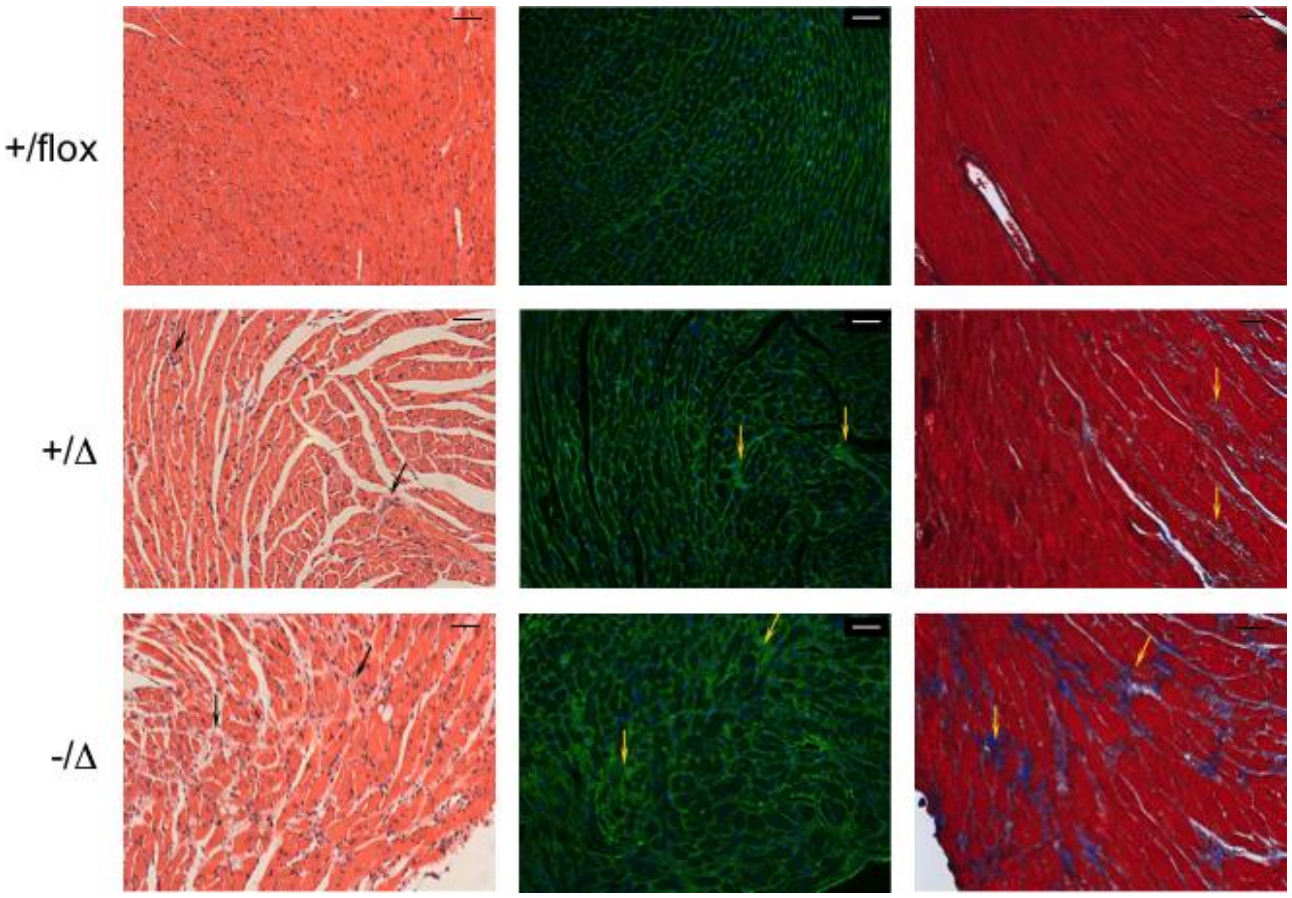


Figure 3

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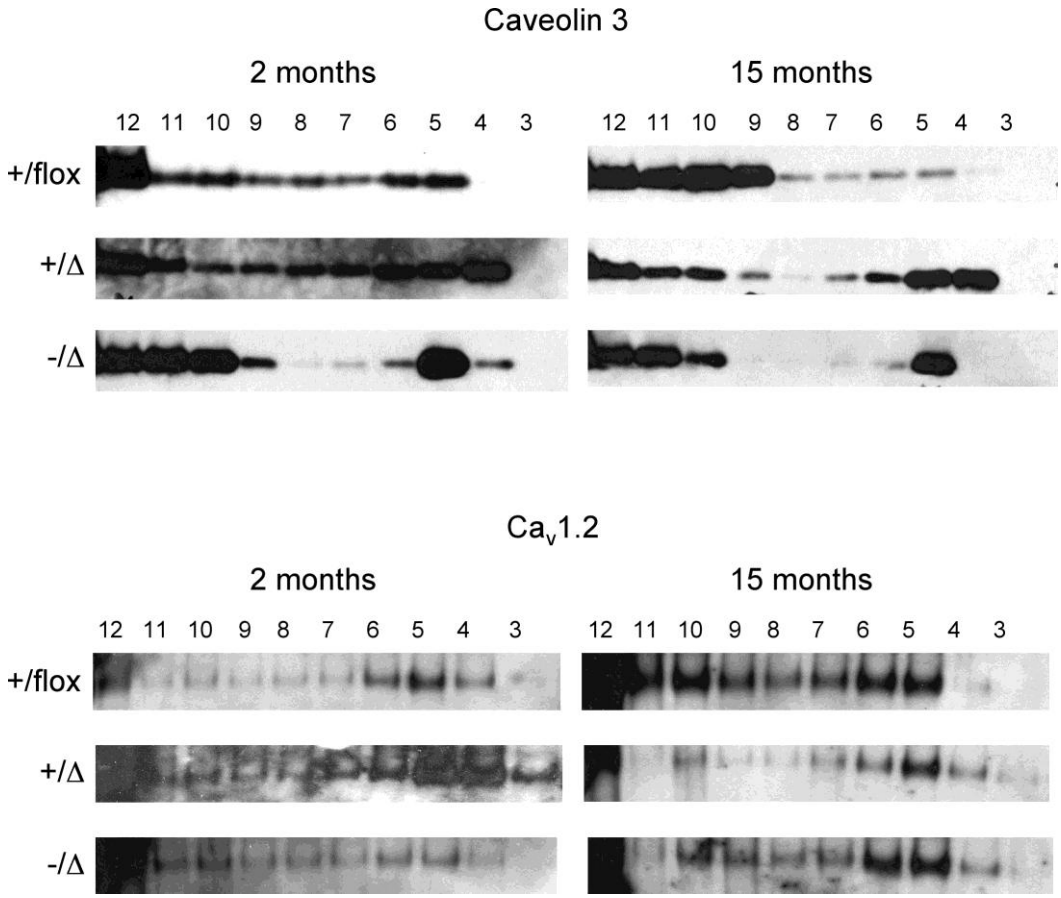


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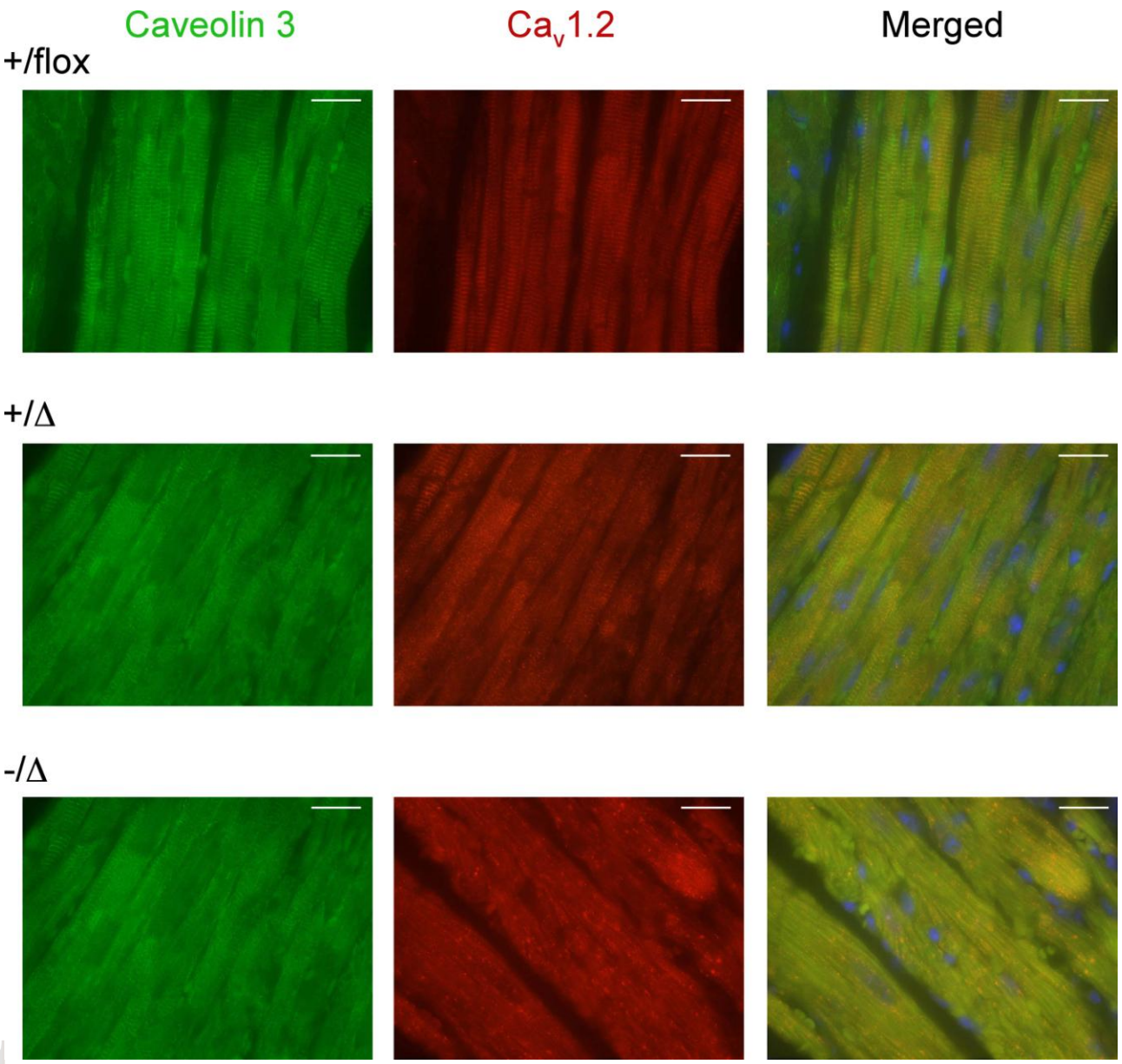


Figure 5

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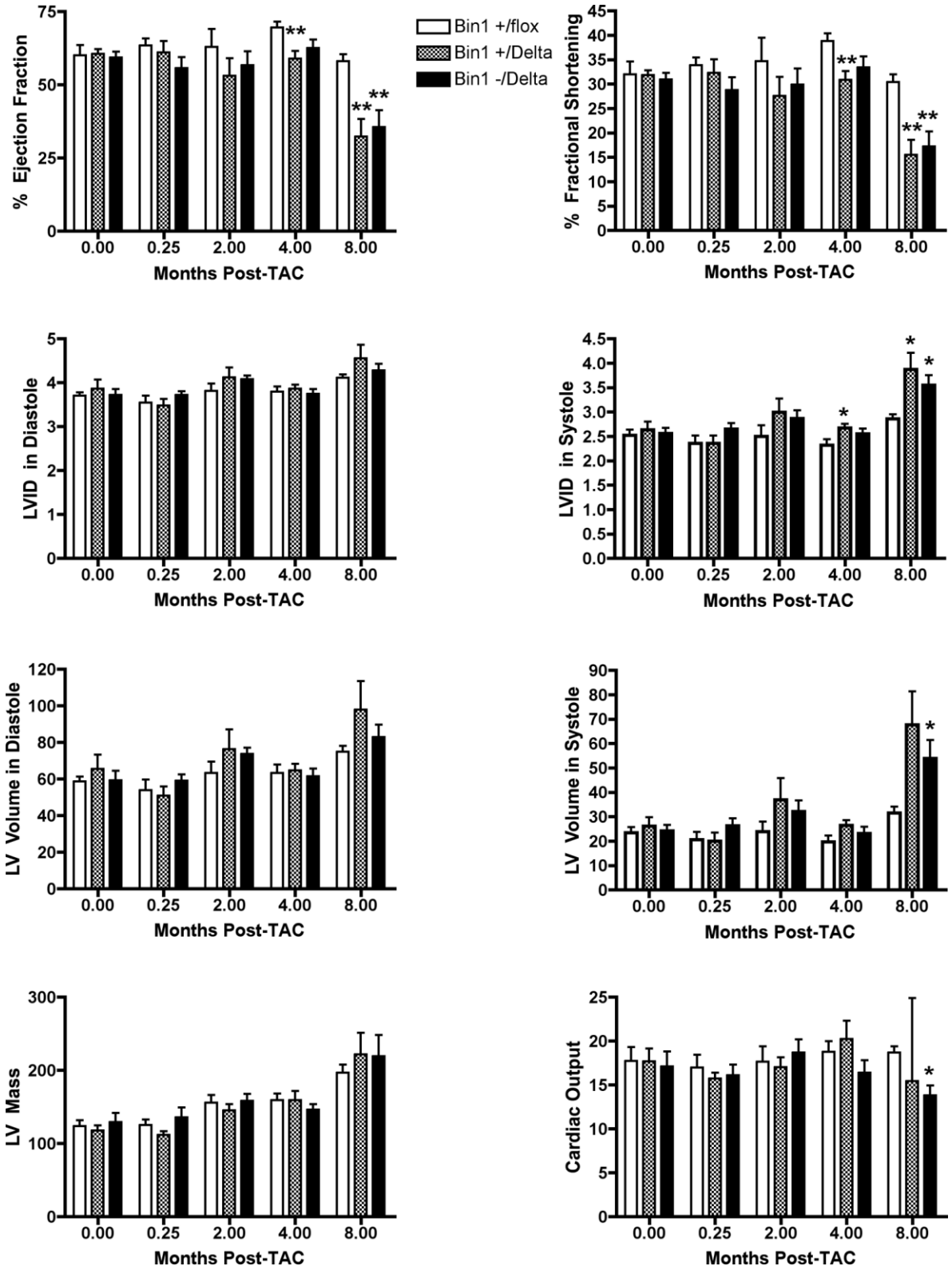


Figure 6

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