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Reversibility of Adverse, Calcineurin-Dependent Cardiac Remodeling

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Abstract

Background—Studies to dissect the role of calcineurin in pathological cardiac remodeling have relied heavily on murine models, where genetic gain- and loss-of-function manipulations are initiated at or before birth. However, the great majority of clinical cardiac pathology occurs in adults. Yet, nothing is known about the effects of calcineurin when its activation commences in adulthood. Further, despite the fact that ventricular hypertrophy is a well established risk factor for heart failure, the relative pace and progression of these two major phenotypic features of heart disease are unknown.

Methods and Results—We engineered mice harboring in cardiomyocytes a constitutively active calcineurin transgene driven by a tetracycline-responsive promoter element. Expression of the mutant calcineurin transgene was initiated for variable lengths of time to determine the natural history of disease pathogenesis, and to determine when, if ever, these events are reversible. Activation of the calcineurin transgene in adult mice triggered rapid and robust cardiac growth with features characteristic of pathological hypertrophy. Concentric hypertrophy preceded the development of systolic dysfunction, fetal gene activation, fibrosis, and clinical heart failure. Further, cardiac hypertrophy reversed spontaneously when calcineurin activity was turned off, and expression of fetal genes reverted to baseline. Fibrosis, a prominent feature of pathological cardiac remodeling, manifested partial reversibility.

Conclusions—Together, these data establish and define the deleterious effects of calcineurin signaling in adult heart and reveal that calcineurin-dependent hypertrophy with concentric geometry precedes systolic dysfunction and heart failure. Furthermore, these findings demonstrate that during much of the disease process, calcineurin-dependent remodeling remains reversible.

Keywords

heart failure; hypertrophy; remodeling

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Conflicts of Interest Disclosures
None

Introduction

Strong epidemiological evidence links left ventricular hypertrophy with adverse cardiovascular events, including heart failure and death[1–4]. Consistent with this, current therapies that improve clinical outcomes are often associated with regression of ventricular hypertrophy[5–7]. However, whereas significant strides have been made recently in elucidating the molecular circuitry governing pathological cardiac remodeling[8], few therapies in clinical use target hypertrophic growth mechanisms directly.

Calcineurin is a cytoplasmic protein phosphatase implicated in the pathogenesis of cardiac hypertrophy and heart failure[9]. Calcineurin dephosphorylates transcription factors of the NFAT (nuclear factor of activated T cells) family, resulting in their translocation to the nucleus and triggering of a complex transcriptional program[10]. Calcineurin also targets other proteins to activate non-transcriptional signaling cascades. Consistent with widespread involvement in heart disease, studies in animal models have demonstrated a critical role for calcineurin in biomechanical stress-induced hypertrophic remodeling[11, 12], and calcineurin activation has been observed in heart failure in humans[13–15].

Studies designed to dissect the role of calcineurin signaling in heart disease have relied, by and large, on genetic manipulations in mice. In this context, gain- and loss-of-function manipulations are typically initiated at or before birth. However, recent studies have highlighted the fact that the effects of mechanisms triggered in adulthood oftentimes differ from that seen when the same mechanism is triggered during development, when a host of other events are taking place[16–19]. Further, the great majority of clinically relevant disease states involving calcineurin occur in adults. However, little is known about the effects of calcineurin activation in adult animal models.

Pathological ventricular remodeling is a dynamic process involving changes in cardiomyocyte size and shape, alterations in gene expression and substrate metabolism, changes in intracellular calcium homeostasis, deposition of excess extracellular matrix, sarcomere recruitment, and more[8]. In many animal models, pathological stress elicits a hypertrophic growth response that culminates ultimately in contractile dysfunction and heart failure[4]. In humans, however, where disease-related stress initiates long after cardiac development is complete, this pathogenic progression has been questioned[20]. Further, the extent to which ventricular hypertrophy is a risk factor for systolic dysfunction may depend on specific phenotypic features, such as whether its geometry is concentric or eccentric[3]. Given all this, it is important to determine the natural history of load-induced remodeling and to tease out molecular events governing the progression of hypertrophy and failure.

In light of recent advances in our understanding of signaling cascades governing pathological cardiac remodeling[21], the hypertrophic growth response *per se* has emerged has a viable therapeutic target[9]. This strategy hinges critically, however, on the notion that pathological remodeling is reversible and not just preventable. Yet, little is known at present regarding the extent to which calcineurin-dependent remodeling is reversible. Finally, clinically relevant anti-remodeling therapies, both pharmacological and mechanical, are only modestly efficacious, and hence it is important to tease out the diseased myocyte's capacity for spontaneous recovery.

Thus, we set out to address three questions: a) what are the effects of calcineurin activation initiated during adulthood; b) is ventricular hypertrophy a requisite precursor of calcineurin-dependent heart failure, and if so, what are its features; and c) is the calcineurin-dependent phenotype reversible in the absence of anti-remodeling therapy? To accomplish this, we engineered mice harboring in cardiomyocytes a constitutively active calcineurin transgene driven by a tetracycline-responsive promoter element. Expression of the mutant calcineurin

transgene was initiated in adulthood for variable lengths of time to determine the natural history of disease pathogenesis and associated molecular and functional events, and to identify markers of disease reversibility.

Materials and Methods

A Detailed Methods section is available in the Online Data Supplement.

Animal Care

Transgenic mice that conditionally express a constitutively active calcineurin mutant[22] specifically in cardiomyocytes were generated using the α-myosin heavy chain promoter and the tetracycline transactivator off promoter system[2324]. Both male and female animals aged 5 to 8 weeks were studied using protocols approved by the animal care and use committee of UT Southwestern Medical Center.

Echocardiography

Echocardiograms were performed using a Sonos 5500 system with a 15 MHz probe, and M-mode and 2D parasternal short axis images were obtained. Left ventricular mass was calculated by the cubed method, as previously described[25].

Immunoblot analyses

Immunoblots were performed on protein lysates prepared from left ventricular tissue. All antibodies used are presented in the Data Supplement.

Quantitative real-time RT PCR

RNA was extracted from snap frozen left ventricular tissue and used to prepare cDNA. Real-time PCR was performed using an ABI 7000 system. All primers used are presented in the Data Supplement.

Histology and cardiomyocyte cross-sectional area

Selected hearts were perfusion fixed in paraformaldehyde and subjected to cutting and staining as described previously[26]. Cardiomyocyte area was measured from hematoxylin and eosin-stained images and from wheat germ agglutinin-stained images. Fibrosis was measured from picrosirius red-stained images. Hydroxyproline analysis was performed based on previously described methods[27].

In vivo electrophysiology studies

In vivo electrophysiology studies were performed on sedated mice using a multipolar electrode catheter placed via the right jugular vein as previously described[28].

Statistical methods and data handling

All data are presented as mean ± standard deviation (SD). Comparison of data between two groups was performed using the Mann Whitney U test. The authors had full access to, and take full responsibility for, the integrity of these data. All authors have read and agree to the manuscript as written.

Results

To test the effects of calcineurin activation in the adult mammalian heart, we generated transgenic mice that conditionally express a constitutively active calcineurin mutant specifically in cardiomyocytes. We used the tetracycline-inhibited transactivator system

under control of the αMHC promoter (αMHC-tTA) to control expression of a constitutively active form of the human calcineurin A subunit (CnA*) (Figure 1A). Several lines of tetO-CnA* mice were generated and then crossed with αMHC-tTA mice. A line of double transgenic mice (tTA/CnA*) was selected for analysis which manifested increased cardiac mass by 8 weeks of age, similar to that previously described for conventional αMHC-calcineurin transgenic mice[10]. For subsequent experiments, all mice including breeding pairs were administered doxycycline via drinking water so that expression of the CnA* transgene was suppressed *in utero* and in early post-natal life.

Doxycycline was withdrawn from several tTA/CnA* mice at age 8 weeks, and hearts were harvested at age 16 weeks. Western blot analysis of protein from ventricular tissue confirmed expression of a truncated calcineurin protein of the expected molecular weight in tTA/CnA* mice (Figure 1B, Supp Figure IA). This protein was not evident in hearts from single transgenic αMHC-tTA mice or from tTA/CnA* mice that continued to receive doxycycline, confirming conditional expression of CnA*.

We investigated a downstream target of calcineurin signaling in order to confirm increased calcineurin activity in tTA/CnA* hearts. The expression of regulator of calcineurin (RCAN1.4) is directly controlled by calcineurin via the NFAT pathway[29, 30]. We observed a robust increase in RCAN1.4 in hearts of tTA/CnA* mice compared to that of single transgenic αMHC-tTA mice (Figure 1C, Supp Figure IA), occurring within 5 days of doxycycline withdrawal (Figure 1D, Supp Figure IB). This augmentation in RCAN1.4 protein levels was not evident when doxycycline was continued in tTA/CnA* mice (Figure 1C). Thus, doxycycline was effective in suppressing the expression and activity of CnA* in double transgenic tTA/CnA* mice.

Calcineurin activation in the adult heart triggers pathological hypertrophy

To test the effects of prolonged calcineurin activation in adult heart, doxycycline was withdrawn from tTA/CnA* mice at 5 weeks of age, and hearts were harvested at 21 weeks of age (Figure 2A). For comparison, a second group of tTA/CnA* mice was maintained on doxycycline to suppress CnA* throughout the study. First, echocardiograms were performed biweekly to assess serial changes in cardiac size and function. Calcineurin signaling in CnA* ON mice triggered a significant decline in fractional shortening consistent with impaired ventricular systolic function (Figures 2B). Calcineurin activation elicited a 34% increase in LV internal diameter at end-diastole (LVIDd OFF 3.23±0.40 mm, n=10; LVIDd ON 4.34±1.07 mm, n=14, $p<0.01$), and an even greater increase in LV internal diameter at end-systole (LVIDs OFF 1.04±0.36 mm, LVIDs ON 2.93±1.30, $p<0.01$, Figures 2B, 2C). The greatest decline in contractile function occurred between 2 to 6 weeks after removing doxycycline. No significant decline in fractional shortening occurred in tTA/CnA* mice that were maintained on doxycycline in the CnA* OFF group.

Necropsy analysis revealed a significant increase in cardiac mass after 16 weeks of calcineurin activation (Figure 2D, 2E), and three animals (of 17) in the CnA* ON group died before study completion (no deaths of 10 mice occurred in the CnA* OFF group). Lung weights were similar between the two groups, although the two CnA* ON mice with the largest hearts were noted to have lung weights twice that of controls.

Cardiomyocyte hypertrophy was evident upon microscopic examination of sections of ventricular tissue. Cardiomyocyte cross-sectional area increased significantly in CnA* ON mice (266 ± 82 μm^2, n=240 cells, 4 hearts) compared to CnA* OFF mice (185 ± 42 μm^2, n=240 cells, 4 hearts, $p<0.01$) (Figure 2F, 2G, Suppl Figure II). We also noted prominent interstitial fibrosis in hearts subjected to 16 weeks of calcineurin activation (Figure 2H).

Quantification of fibrosis from histological images revealed more fibrosis in hearts of CnA* ON mice (ON 6.0±1.5%, n=5; OFF 3.2±2.0%, n=6; p=0.02).

We next set out to determine the phenotype of calcineurin-induced ventricular remodeling after just 8 weeks of calcineurin activation (Figure 3A). Single transgenic αMHC-tTA littermates were used as controls, and all mice were subjected to the same schedule of doxycycline. Hearts were harvested 8 weeks after doxycycline removal, a time point associated with significant declines in fractional shortening based on our initial study (Figure 2C). Eight weeks of calcineurin activation in adult hearts triggered profound pathological hypertrophy (Figure 3). Analysis of echocardiographic images revealed significant declines in systolic function (Figure 3B), robust increases in ventricular mass, left ventricular wall thickness, and chamber dimension (Figure 3C). Of note, we observed a subtle increase in cardiac mass in single-transgenic tTA mice compared to wild-type littermates, but there was no significant decline in fractional shortening (Supp Figure III). We, therefore, used single-transgenic tTA mice as controls for comparison to double-transgenic mice. [Head-to-head comparisons of LV mass estimated by echo and measured on necropsy revealed high correlation, Supp Figure IV]

Post-mortem analysis revealed consistent results; heart weight:body weight ratios increased 48% ($p<0.05$) in tTA/CnA* mice compared to hearts from control mice (Figure 3D). To evaluate for activation of the fetal gene program, a marker of pathological remodeling in heart, we extracted protein and RNA from ventricular tissue 8 weeks after removing doxycycline. Calcineurin signaling triggered dramatic increases in β myosin heavy chain (βMHC) protein and mRNA (Figure 3E, 3F, Supp Figure IC). There was also a significant increase in BNP expression, but no statistically significant changes in ANF or SERCA2a expression were observed.

Calcineurin-induced ventricular hypertrophy precedes systolic dysfunction

In animal models where calcineurin is activated early in life, hypertrophic growth of the heart precedes contractile dysfunction and heart failure. In humans, however, where disease-related stress initiates long after cardiac development is complete, this pathogenic progression has been questioned[20]. Does ventricular hypertrophy progress routinely to heart failure, or does ventricular hypertrophy develop secondarily, as a consequence of systolic dysfunction? Further, the extent to which ventricular hypertrophy is a risk factor for systolic dysfunction may depend on specific phenotypic features, such as whether it is concentric or eccentric[3]. To test these important questions, we studied 8-week old male mice with serial echocardiograms performed biweekly during calcineurin activation. First, we measured left ventricular mass and wall thickness by echocardiogram, observing robust increases within two weeks (Figure 4A, 4B). Next, we evaluated ventricular size and performance. Interestingly, there was no significant decrease in fractional shortening at two weeks despite the development of ventricular hypertrophy (Figure 4C). Fractional shortening was significantly decreased by four weeks, but left ventricular chamber dimension remained unchanged until 8 weeks (Figure 4D). Thus, calcineurin activation in adult mice triggered ventricular hypertrophy followed by systolic dysfunction, and left ventricular chamber dilation occurred last. There was no significant difference in heart rates in these mice at the time the echocardiograms were performed (Supp Figure V).

In order to evaluate the relationship between gene expression and ventricular remodeling, we collected RNA from mice at one week and eight weeks after removing doxycycline. Just one week after removing doxycycline, we observed a robust increase in RCAN1.4 expression consistent with activation of calcineurin (Figure 4E). Immunoblots for RCAN1.4 also confirmed robust calcineurin activation at one week (data not shown). There was, however, no significant change in expression of fetal gene markers at this early time point.

After eight weeks of calcineurin activation, however, βMHC and BNP expression had increased significantly (Figure 4F). These data, then, demonstrate that calcineurin activation alone does not provoke an immediate increase in βMHC or BNP expression. Rather, calcineurin activation may trigger a cascade of molecular signaling and remodeling events that eventually results in increased expression of fetal gene markers.

Adverse remodeling regresses after activated calcineurin is turned off

Adults with heart disease often present to medical attention with significant hypertrophy and/or systolic dysfunction. However, the extent to which calcineurin-dependent adverse remodeling is reversible in the absence of therapy is unknown. To test this, doxycycline was withdrawn from a group of tTA/CnA* mice for 8 weeks in order to activate calcineurin signaling; doxycycline was subsequently restored for an additional 8 weeks (Figure 5A). In these ON/OFF mice, ventricular function declined during the first 8 weeks similar to that of the ON group of mice (Figure 5B). Then, after doxycycline was restored, ventricular function began to improve, reaching statistical significance at 10 weeks (2 weeks after restarting doxycycline) (Figure 5B). This recovery of ventricular function occurred without the assistance of pharmacological therapies for heart failure. At study completion, the average cardiac mass for mice in the ON/OFF group was significantly less than that of mice in the ON group subjected to 16 weeks of persistent CnA* expression (Figure 5C). There was variability in the degree of reverse remodeling, but no specific parameters of ventricular hypertrophy or chamber dimension were predictive of the extent of reverse remodeling (Online Supplement, Supp Fig VI, Supp Fig VII).

To evaluate the kinetics of extracellular matrix deposition during the course of calcineurin-dependent ventricular remodeling, we quantified ventricular collagen accumulation by hydroxyproline assay. Collagen content within the LV did not increase significantly after 8 weeks of calcineurin activation (ON 0.9±0.2 mg/g, n=3; OFF 0.9±0.2, n=3; p=NS) despite significant ventricular dilatation and declines in systolic function (Figure 5). By contrast, after 16 weeks of calcineurin signaling, collagen content was significantly increased both by hydroxyproline assay (ON 1.6±0.1 mg/g, n=2; OFF 0.80±0.05, n=2, $p<0.05$) and analysis of picrosirius red-stained images (ON 6.0±1.5%, n=5; OFF 3.2±2.0%, n=6; p=0.02). Collagen content in hearts of ON/OFF mice was similar to control levels (ON/OFF 4.0±2.0%, n=6). Surprisingly, we were unable to induce ventricular tachyarrhythmia in ON mice despite the presence of significant interstitial fibrosis (Online Supplement, Supp Fig VIII). In aggregate, these findings suggest that ventricular fibrosis is a relatively late-arriving feature of the adverse remodeling phenotype, developing after both ventricular hypertrophy and altered contractile function are present.

From these data, it is unclear whether cardiac mass decreased when calcineurin signaling was extinguished or whether hearts in ON/OFF mice simply stopped growing when CnA* was turned off. To test this, we performed two additional, parallel experiments on tTA/CnA* mice. For study A, CnA* was activated at age 8 weeks, and mice were sacrificed at age 16 weeks. For study B, CnA* was also activated at age 8 weeks, echocardiograms were performed at age 16 weeks to confirm depressed ventricular function, and CnA* was suppressed until age 24 weeks at which time mice were sacrificed. As in the previous experiments, echocardiography confirmed recovery of ventricular function and a decrease in LV mass when CnA* was turned off in Study B (Figure 6A, 6B). Analysis of protein from left ventricular tissue confirmed robust activation of calcineurin in tTA/CnA* hearts at the time of sacrifice in study A (Figure 6C). By contrast, calcineurin activity was not elevated in tTA/CnA* hearts at study completion in study B, confirming successful suppression of CnA* during the second half of the experiment. Importantly, heart weights at the completion of study B were significantly lower than those at the completion of study A, despite having been subjected to the same 8 weeks of CnA* activation (Figure 6D).

Activation of the genes coding for βMHC and BNP was detected after 8 weeks of calcineurin activation (Figure 3E). After 8 weeks of recovery in study B, βMHC and BNP mRNA returned to control levels (Figure 6E). Interestingly, those mice with limited recovery of fractional shortening (less than 50% at after 8 weeks of recovery) also had normal expression of βMHC (included in Figure 6E). Conversely, BNP expression remained elevated in some but not all mice with limited recovery.. Together, these data lend strong support to the concept that calcineurin-dependent remodeling of the heart can be reversed even without anti-remodeling therapy.

Discussion

In recent years, calcineurin has emerged as a major mechanism of pathological cardiac remodeling, active in numerous forms of heart disease. As such, this molecule has generated much interest as a target of therapy. Here, we addressed several questions relevant to the therapeutic targeting of calcineurin in heart disease: what are the effects of calcineurin activation in the fully developed adult heart; does calcineurin-dependent hypertrophy precede contractile dysfunction, and, if so, what are its phenotypic features; to what extent are these phenotypes reversible; can reversal be accomplished in the absence of therapy? To accomplish this, we engineered a mouse model that provides high fidelity, spatiotemporal control of calcineurin activation. From these experiments, we report the following significant findings: a) calcineurin activation in adulthood triggers robust pathological remodeling; b) ventricular hypertrophy, concentric in nature, precedes systolic dysfunction and tissue fibrosis; c) ventricular fibrosis develops after both hypertrophy and contractile dysfunction have arisen; d) hypertrophic growth, and contractile dysfunction are each partially and spontaneously reversible. Together, these findings shed new light on the adverse remodeling response elicited by calcineurin and raise yet further the prospects of targeting this molecule for therapeutic gain.

Calcineurin signaling is maladaptive in adult heart

Heart disease is the leading cause of morbidity and mortality in the industrialized world, contributing importantly to ever-expanding health care expenditures[31]. The pathogenesis of many forms of heart disease involves a set of complex remodeling processes, many of which are independent risk factors for adverse clinical events[8, 32]. For example, the presence of left ventricular hypertrophy in patients with hypertension is associated with substantially increased morbidity and mortality[1]. Also, a decline in ejection fraction after myocardial infarction is a major predictor of increased risk of developing heart failure or sudden death[33]. In many of these events, persistent or excessive activation of the calcineurin signaling pathway is thought to play a significant role[21].

Previous models of calcineurin activation in neonatal murine hearts demonstrated an almost 3-fold increase in cardiac mass at only 3 to 12 weeks of age[10]. Working with adult myocytes, we observed significantly less hypertrophy despite the presence of robust calcineurin activation for 16 weeks. In addition, adult mice manifested less than 10% mortality at 16 weeks, which contrasts with the greater than 50% mortality at 16 weeks observed when calcineurin is activated in neonatal mice[34]. Together, these findings are consistent with a model where pathological signaling events activated early in life interact with developmental pathways, often compounding the resulting adverse phenotype.

Our model of conditional activation of calcineurin provided an opportunity to investigate the time course of events that occur with calcineurin signaling. We found that ventricular hypertrophy is an early event in the progression of calcineurin-induced heart failure. Left ventricular mass, measured noninvasively by echocardiography, was significantly increased after just 2 weeks of calcineurin activation, while left ventricular cavity size and ejection

fraction remained normal. Conversely, we found that tissue fibrosis and fetal gene activation are late-appearing aspects of the phenotype, emerging only after significant contractile dysfunction has developed. A dramatic increase in βMHC expression was apparent after several weeks of calcineurin activation. Despite an increase in RCAN1.4 during the first week, confirming calcineurin activation, we did not observe a significant increase in βMHC at this early time point. This suggests that calcineurin triggers a cascade of signaling events that later leads to the rise in βMHC. Yet, when calcineurin was turned off, βMHC returned to control levels. The contribution of βMHC expression to systolic dysfunction remains uncertain.

These findings lend further support to the notion that calcineurin-induced hypertrophy is maladaptive, as a decline in systolic function ultimately developed in these mice without any additional stressors, such as myocardial infarction or pressure overload. Furthermore, these data are consistent with previous findings that inhibition of calcineurin-dependent signaling in the setting of pressure overload blunts hypertrophy without adverse effects on cardiac function or clinical events[9, 35].

Calcineurin as a therapeutic target

While preventive strategies will undoubtedly remain cornerstones of therapy, many patients do not present to clinical attention until after significant structural heart disease has already developed. It is therefore critical to parse the various pathological elements and determine and quantify their potential for reversal. Some, such as myocyte death, have essentially no potential for recovery. Others, however, such as cellular hypertrophy, contractile dysfunction, and fibrosis hold potential for significant reversibility. Prior to this study, however, nothing was known about the kinetics of development of the multiple phenotypes elicited by calcineurin, their emergence in adult heart, and their potential for reversal.

Both pharmacological and device-based therapies improve clinical outcomes in association with reverse remodeling[5, 36, 37], and calcineurin signaling mechanisms may prove to be new targets for recovery of cardiac function. For example, calcineurin suppression by systemic administration of cyclosporine A is capable of reversing cardiac hypertrophy[38], although cyclosporine A may trigger hypertrophy in some models of heart disease[39]. Rapamycin treatment will reverse hypertrophy induced by pressure overload[40, 41] and in AKT transgenic mice[42]. Our results here extend these observations by demonstrating the reversibility of some of the effects of calcineurin signaling. Further, that this reversal occurs when myocyte-specific calcineurin activity is suppressed points to a cell-autonomous mechanism of reverse remodeling.

Variable degrees of reverse remodeling were observed in ON/OFF mice, but left ventricular geometry did not emerge as a robust predictor of the potential for recovery of function; mice with the greatest LV dilation or lowest percent fractional shortening following 8 weeks of calcineurin activation sometimes demonstrated significant reverse remodeling when calcineurin activation was turned off. Thus, other factors may mark, or even determine, the potential for recovery of ventricular function. Interstitial fibrosis emerged as a late manifestation of calcineurin-triggered remodeling, possibly the result of activation of secondary signaling cascades. Excessive fibrosis in some hearts may have limited reverse remodeling when calcineurin signaling was extinguished.

A major objective of our study was to determine whether hypertrophy was reversible if the underlying cause were removed. As some evidence suggests that the capacity for cardiac plasticity and repair declines age, we chose to study young adult mice in an effort to obviate confounding, age-dependent changes in plasticity. In other words, we set out specifically to study young adults – with presumably maximal capacity for reversibility – in order to

determine whether such reversibility is possible in the setting of a robust signaling cascade (*viz.* calcineurin). Moving forward, having established for the first time that such reversibility of remodeling is, indeed, possible, it will be of interest to test for age-dependent declines in cardiac plasticity.

Increased calcineurin activity has been reported in the hearts of patients with multiple forms of structural heart disease[13–15]. Further, inhibition of calcineurin via either pharmacological or transgenic strategies blunts hypertrophic growth in a variety of animal models of heart disease[11, 12, 35, 43]. Importantly, suppression of hypertrophic growth by calcineurin inhibition is not associated with impairment of cardiac function or increased mortality[9]. Consistent with this, data reported here demonstrate that calcineurin-induced cardiac hypertrophy results, in fact, in impaired ventricular function. These findings, then, add to a growing literature implicating the hypertrophic growth response *per se* as a maladaptive response to stress and a viable target for therapeutic intervention[9].

Conclusion

Calcineurin activation in the adult heart triggers pathological remodeling which is, at least in part, reversible. Whereas certain forms of hypertrophic growth are thought to represent compensatory responses to environmental stress, calcineurin-induced remodeling appears to be uniquely maladaptive, progressing ultimately to systolic dysfunction, heart failure, and premature mortality. This progression, however, is reversible, suggesting that novel calcineurin-targeting therapies may prove effective in further improving outcomes and quality of life for patients with heart disease.

Novelty and Significance

What is Known?

- Left ventricular hypertrophy is associated with adverse cardiovascular events, including heart failure and death.
- Calcineurin is a cytoplasmic protein phosphatase implicated in the pathogenesis of cardiac hypertrophy.

What New Information Does This Article Contribute?

- Calcineurin signaling in the adult heart triggers ventricular hypertrophy with markers of pathological remodeling.
- Calcineurin-induced ventricular hypertrophy precedes the development of systolic dysfunction and heart failure.
- Calcineurin-induced cardiac hypertrophy reverses when calcineurin signaling is turned off.
- Fetal gene expression and ventricular fibrosis, each a late manifestation of pathological remodeling, manifest significant reversibility.

Strong epidemiological evidence links left ventricular hypertrophy with adverse cardiovascular events, and heart failure therapies that improve clinical outcomes are often associated with regression of hypertrophy. Calcineurin may play a role in the pathogenesis of hypertrophic heart disease, yet little is known regarding the mechanisms of calcineurin-induced ventricular hypertrophy in the adult heart. Data reported here demonstrate that calcineurin signaling in adult cardiomyocytes triggers pathological ventricular hypertrophy and heart failure. Hypertrophy, heart failure, and other aspects of the pathological remodeling response reverse when calcineurin activation is eliminated.

This study is significant for defining the time course and natural history of remodeling events in adult heart triggered by calcineurin signaling. Ventricular hypertrophy precedes the development of systolic dysfunction and heart failure. A robust increase in beta myosin heavy chain expression occurs, but this is not apparent during the first week of calcineurin signaling. Beta myosin heavy chain expression returns to baseline levels as calcineurin-induced hypertrophy reverses. Future studies are warranted to investigate calcineurin and its downstream effectors as therapeutic targets to prevent, and possibly reverse, relevant features of the pathologically remodeled ventricle.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Non-standard Abbreviations and Acronyms

NFAT	nuclear factor of activated T cells
αMHC	alpha myosin heavy chain
βMHC	beta myosin heavy chain
tTA	tetracycline transactivator
CnA	calcineurin A
RCAN	regulator of calcineurin
LVIDd	left ventricular internal diameter at end-diastole
LVIDs	left ventricular internal diameter at end-systole
LVPWd	left ventricular posterior wall thickness at end-diastole
IVSd	interventricular septal thickness at end-diastole

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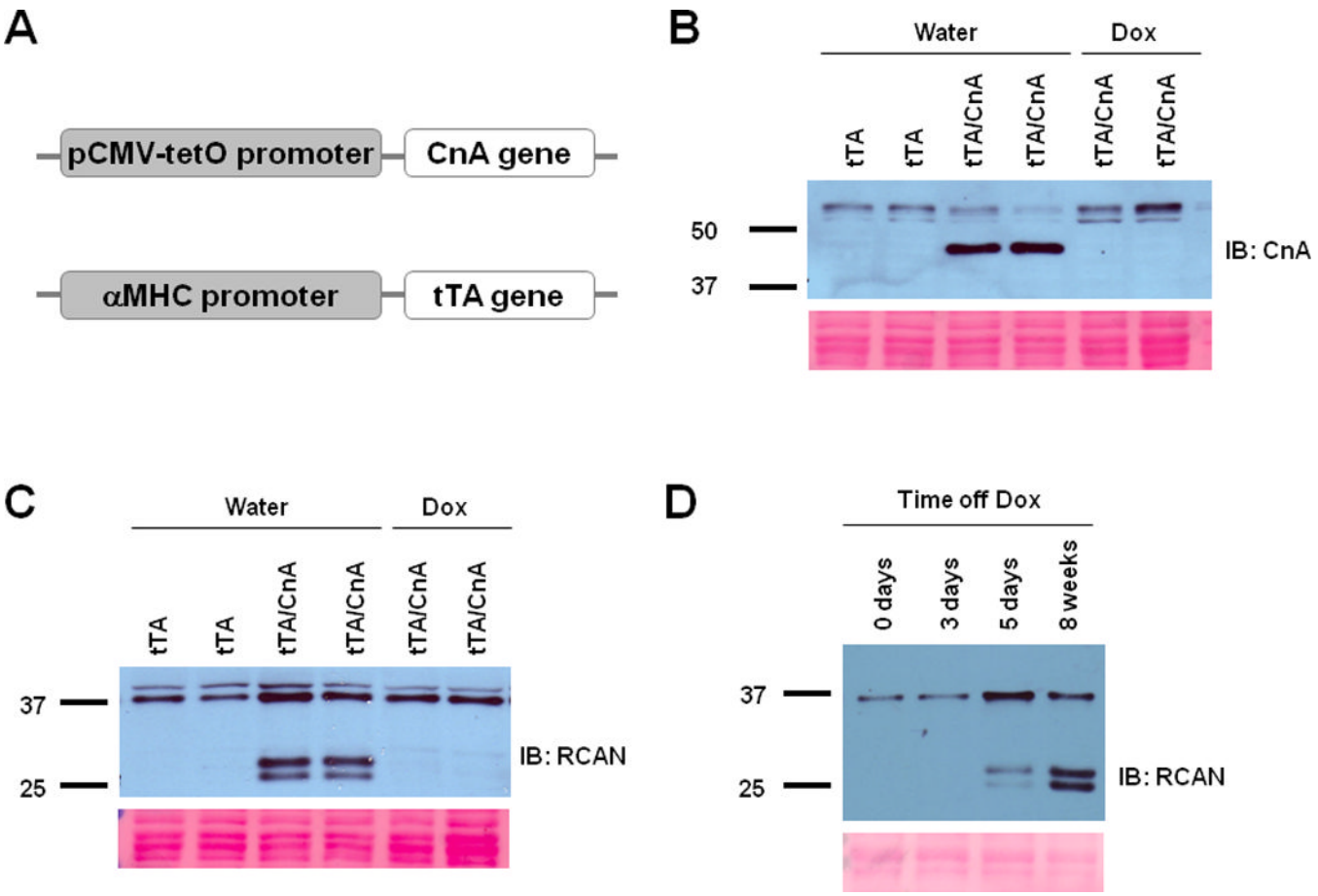
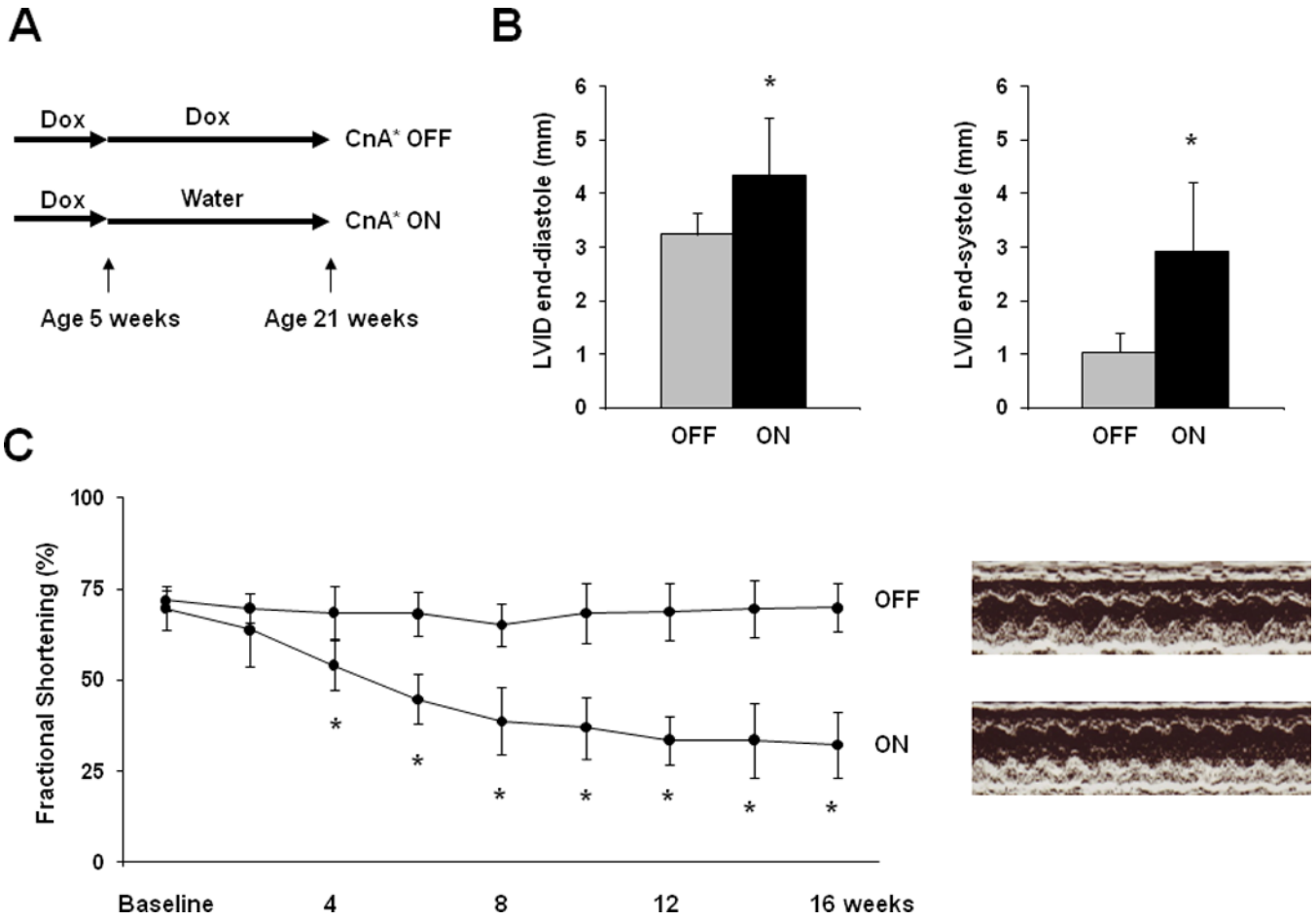


Figure 1. Conditional activation of calcineurin in cardiomyocytes

A: Schematic depiction of the transgenic constructs used to engineer double transgenic tTA/CnA* mice. B, C: Representative immunoblots of LV lysates prepared after 8 weeks of treatment with doxycycline (Dox) or water and probed for calcineurin (CnA) (B) (upper bands represent endogenous calcineurin; lower bands represent mutant calcineurin) or RCAN (C) (lower bands represent RCAN1.4 in different phosphorylation states). Ponceau stains show protein loading in each lane. D: Representative immunoblot of LV lysates probed for RCAN from hearts harvested at different times after removal of doxycycline. tTA, single transgenic for αMHC-tTA only; tTA/CnA, double transgenic for both αMHC-tTA and tetO-CnA*.



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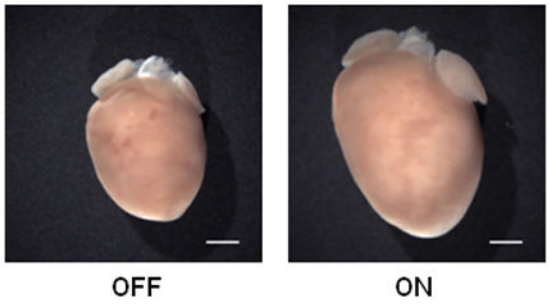

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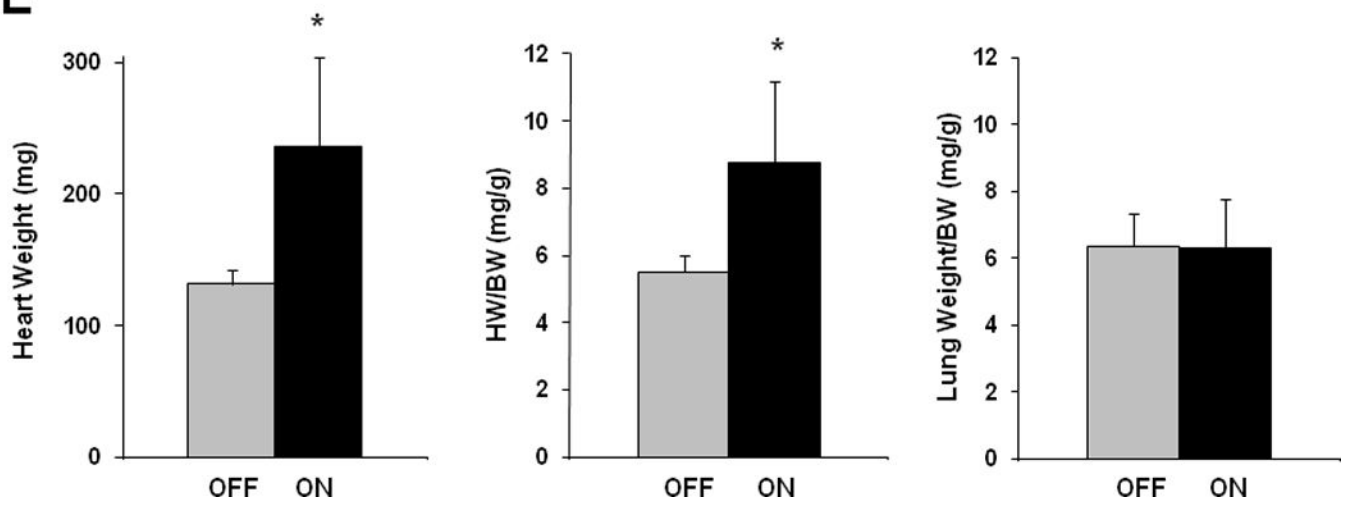


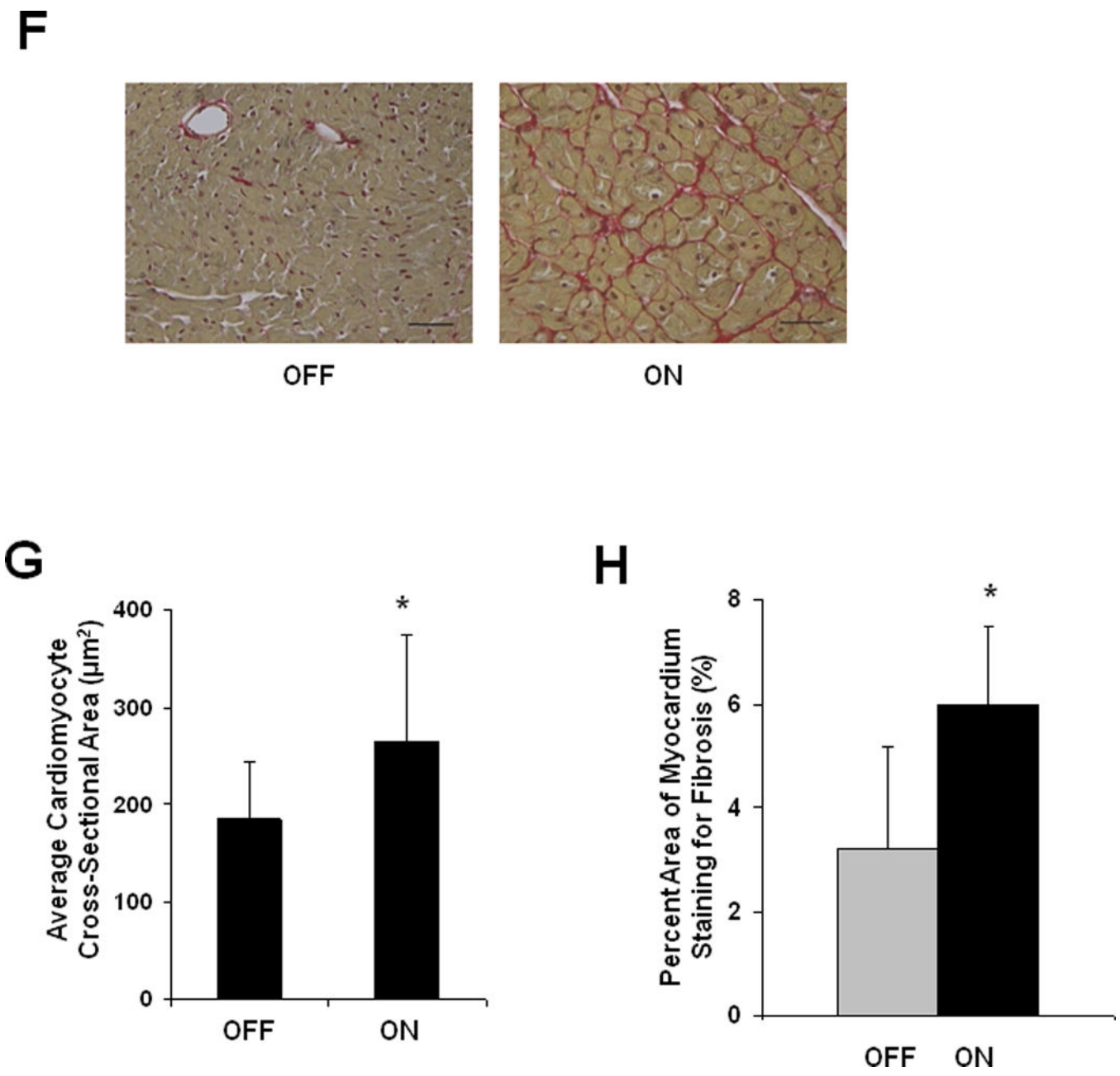
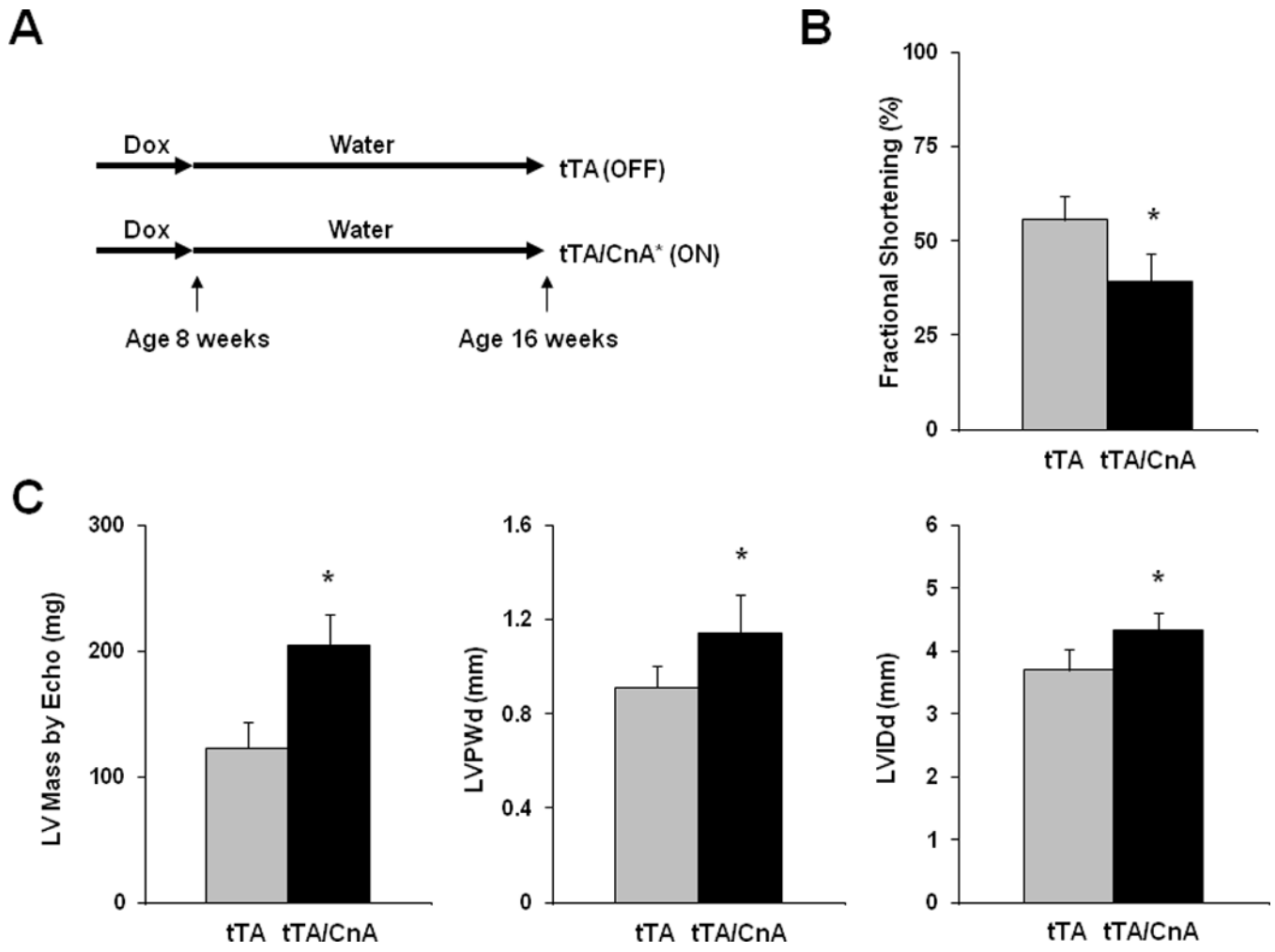


Figure 2. Hypertrophy and heart failure develop when doxycycline is withheld from tTA/CnA* mice

A: Experimental design. Double transgenic tTA/CnA* mice maintained on doxycycline (OFF) were used as controls. B: Left ventricular internal diameter (LVID) at end-diastole and end-systole after 16 weeks of calcineurin activation. C: Mean percent fractional shortening measured from M-mode tracings from mice in the OFF (n=10) and ON (n=14) groups over the course of the experiment. Representative M-mode echocardiographic tracings showing left ventricular mechanical activity at study completion. D: Representative images of hearts at study completion (scale bar = 800 μm). E: Mean absolute heart weight, heart weight normalized to body weight (HW/BW), and lung weight normalized to body weight for mice in the OFF (n=10) and ON (n=14) groups at study completion. F: Representative histological images of picrosirius red staining revealing cardiomyocyte size

and fibrosis at study completion. Bar = 80 μm. G: Mean cardiomyocyte cross-sectional area measured from hearts in the OFF and ON groups (4 hearts per group; 60 cells measured per heart; bar = 1 SD). *$p<0.01$. H. Mean percent area of myocardium staining for fibrosis in the OFF and ON groups (6 images per heart, 6 hearts per group).



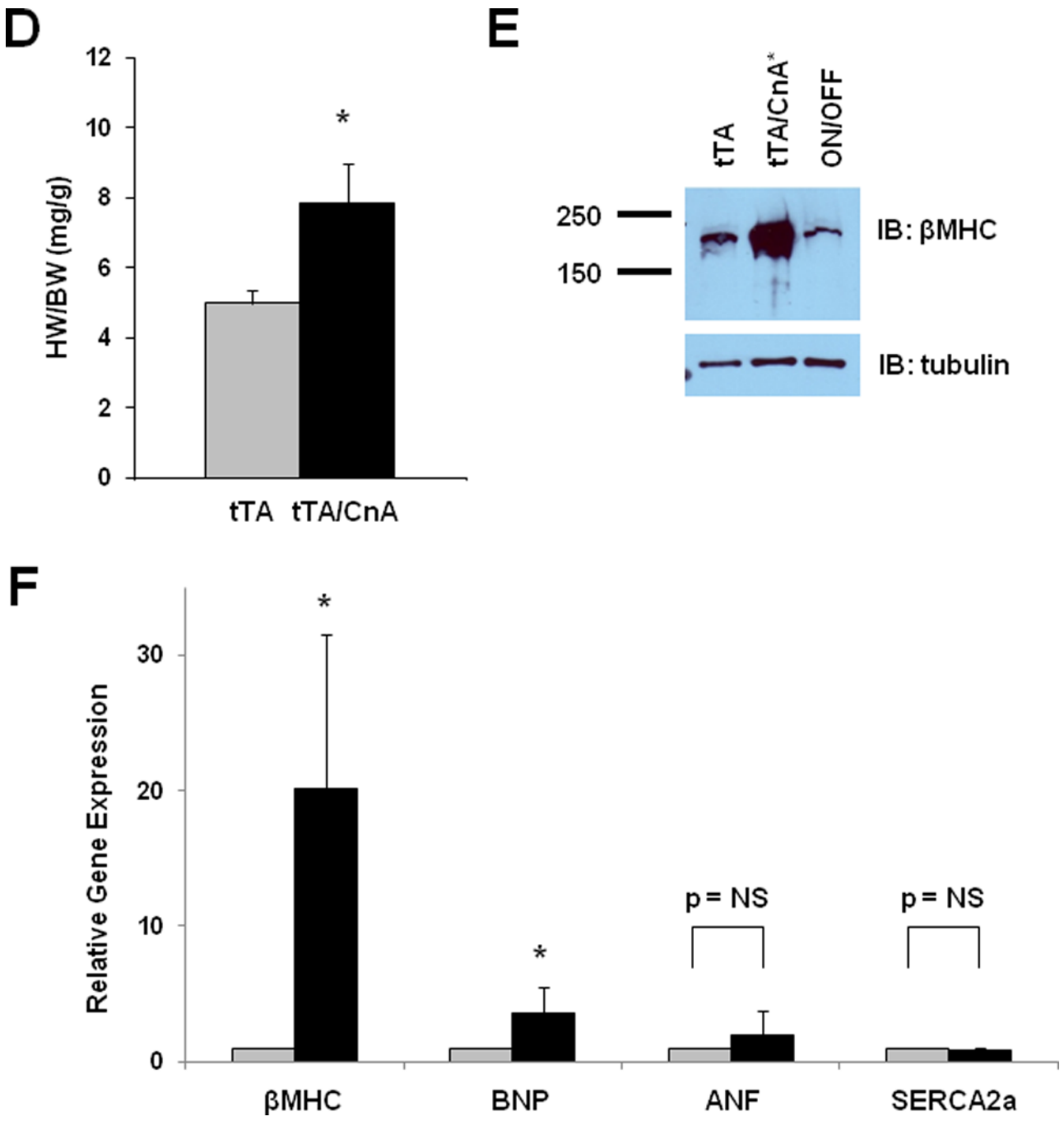
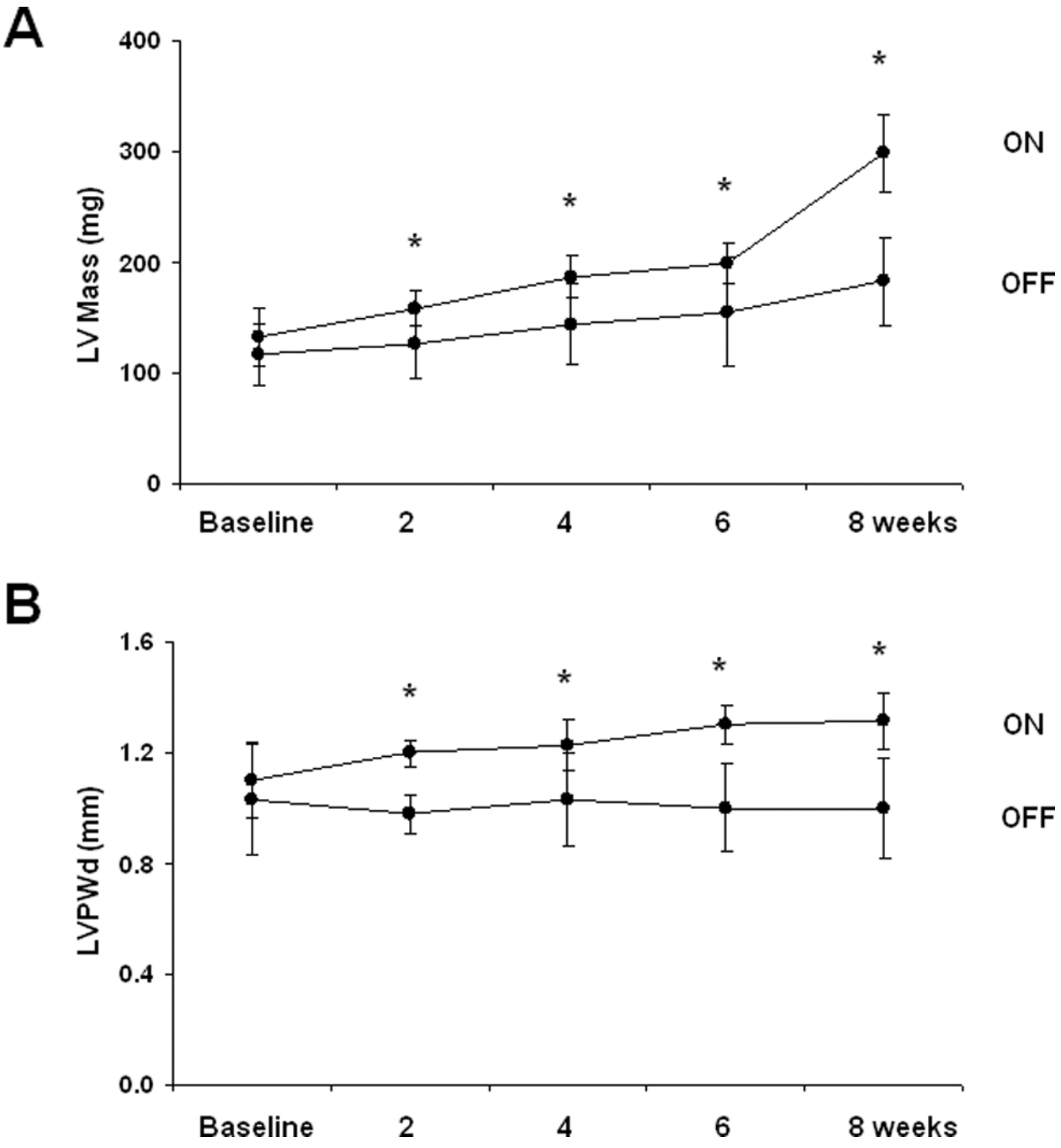
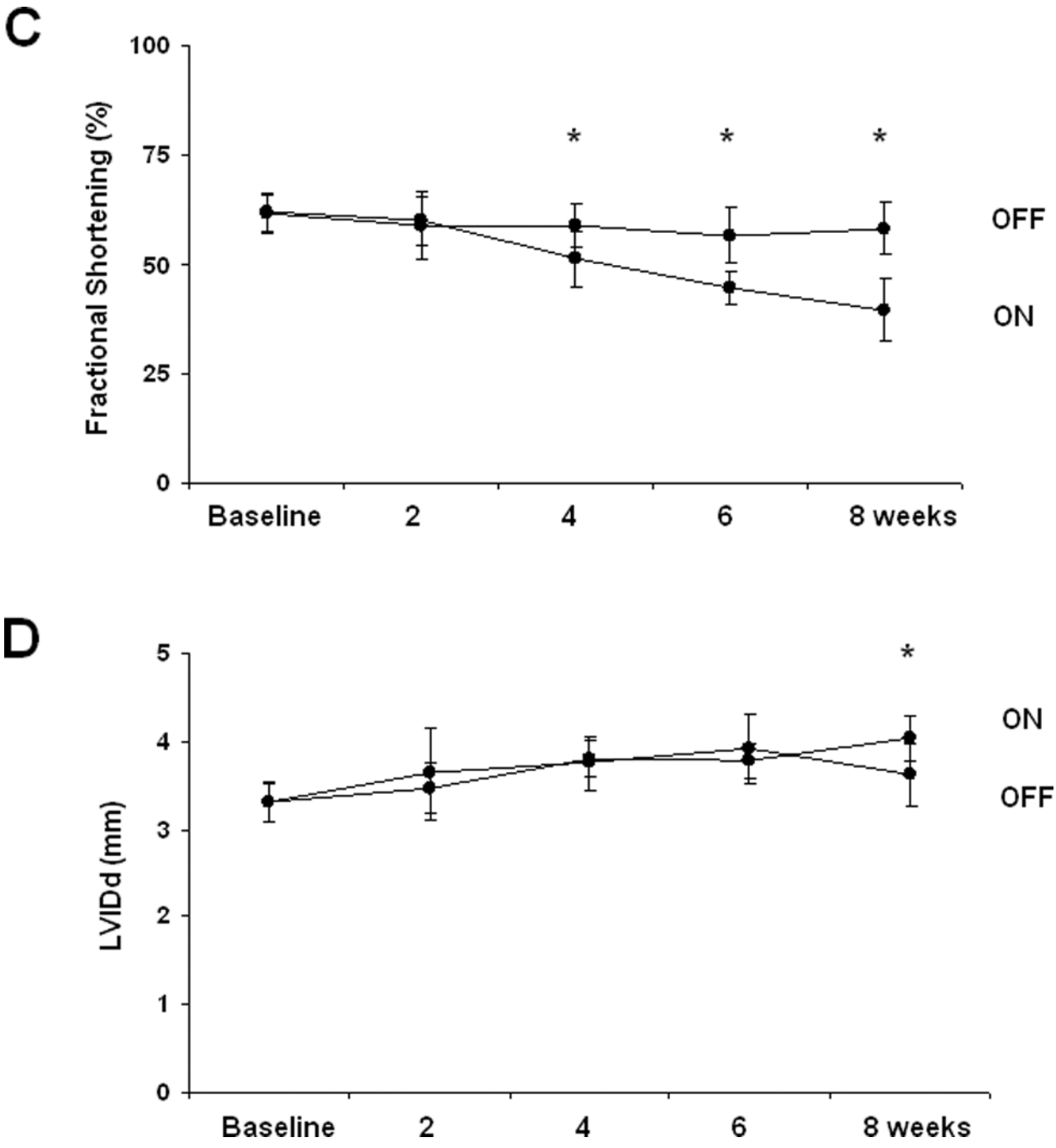


Figure 3. Calcineurin activation in the adult heart triggers pathological hypertrophy

A: Experimental design. Single transgenic tTA mice were used as controls. B: Mean fractional shortening calculated from M-mode tracings for tTA (n=9) and tTA/CnA* (n=11) mice at study completion. C: Mean LV mass, LV posterior wall thickness (LVPWd), and LV internal diameter in diastole (LVIDd) measured from 2D echocardiographic images at study completion. D: Mean heart weight normalized to body weight (HW/BW) measured at necropsy. E: Representative immunoblot of LV lysates from tTA, tTA/CnA*, and ON/OFF hearts probed for βMHC. F: Mean mRNA levels of βMHC, BNP, ANF, and SERCA2a determined by real-time RT-PCR from RNA isolated from tTA (gray bars; n=6) and tTA/CnA* (black bars, n=8) hearts. mRNA levels are calculated relative to cyclophilin mRNA

and normalized to control levels. *p < 0.05 ON: double-transgenic tTA/CnA mice; OFF: single transgenic tTA mice. ON/OFF: double-transgenic tTA/CnA mice after doxycycline was withdrawn for 8 weeks and then restored for 8 weeks.





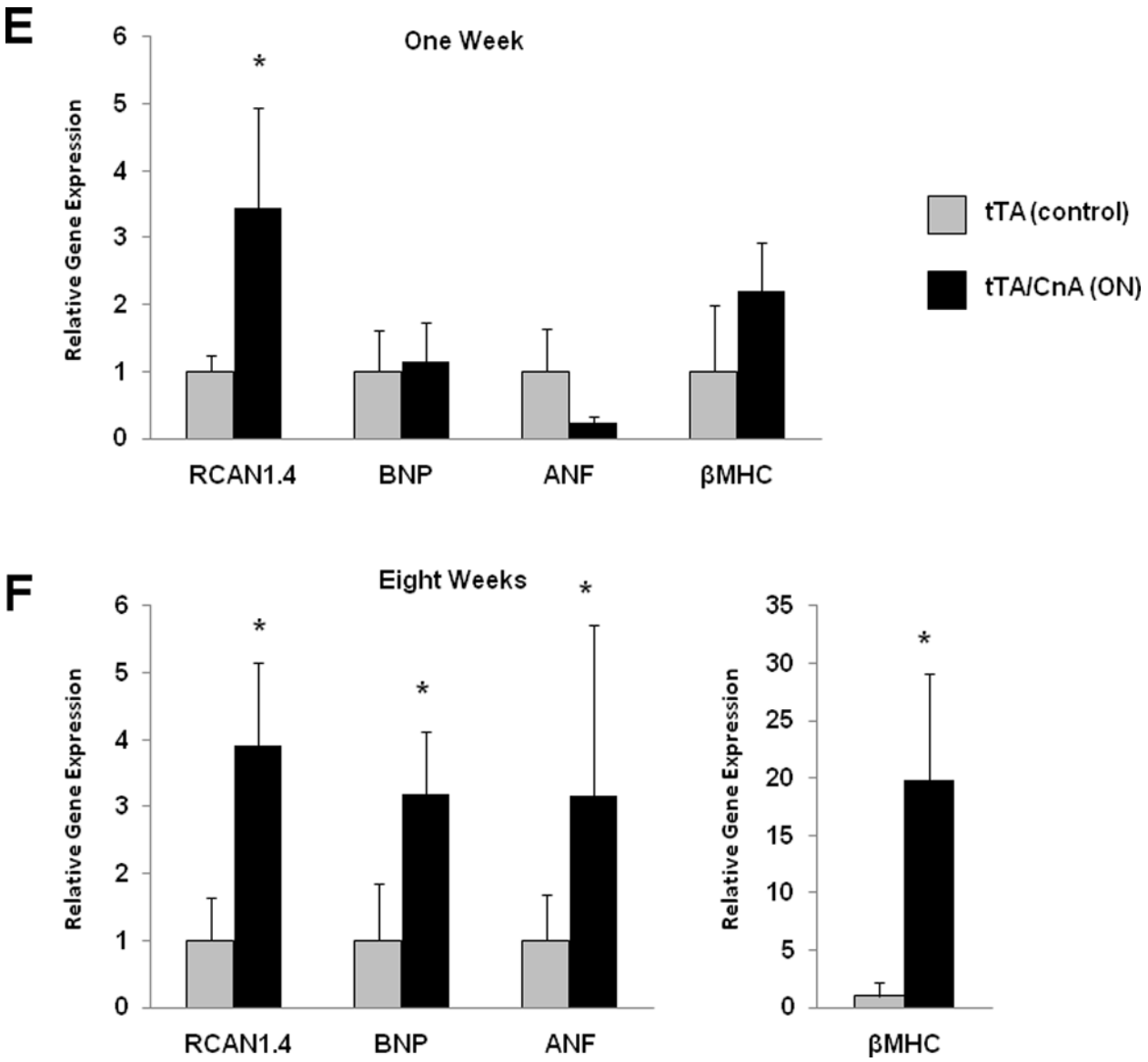


Figure 4. Calcineurin-induced ventricular hypertrophy precedes systolic dysfunction, and fetal gene activation is delayed after calcineurin activation

A: Mean LV mass calculated from serial 2D echocardiographic images of tTA (n=9) and tTA/CnA* (n=9) mice recorded after removing doxycycline. B: Mean LV posterior wall thickness at end-diastole (LVPWd) by serial echocardiography after removing doxycycline. C: Mean percent fractional shortening from serial M-mode tracings obtained after removing doxycycline. D: Mean LV internal diameter in diastole (LVIDd) measured from serial 2D echocardiographic images obtained after removing doxycycline. E,F: Quantitative real-time RT-PCR measurements of mRNA levels of indicated genes from RNA isolated from selected hearts at (E) one week and (F) eight weeks after removing doxycycline. mRNA levels are calculated relative to cyclophilin mRNA and normalized to control levels. *$p < 0.01$. ON: double-transgenic tTA/CNA mice; OFF: single transgenic tTA mice.

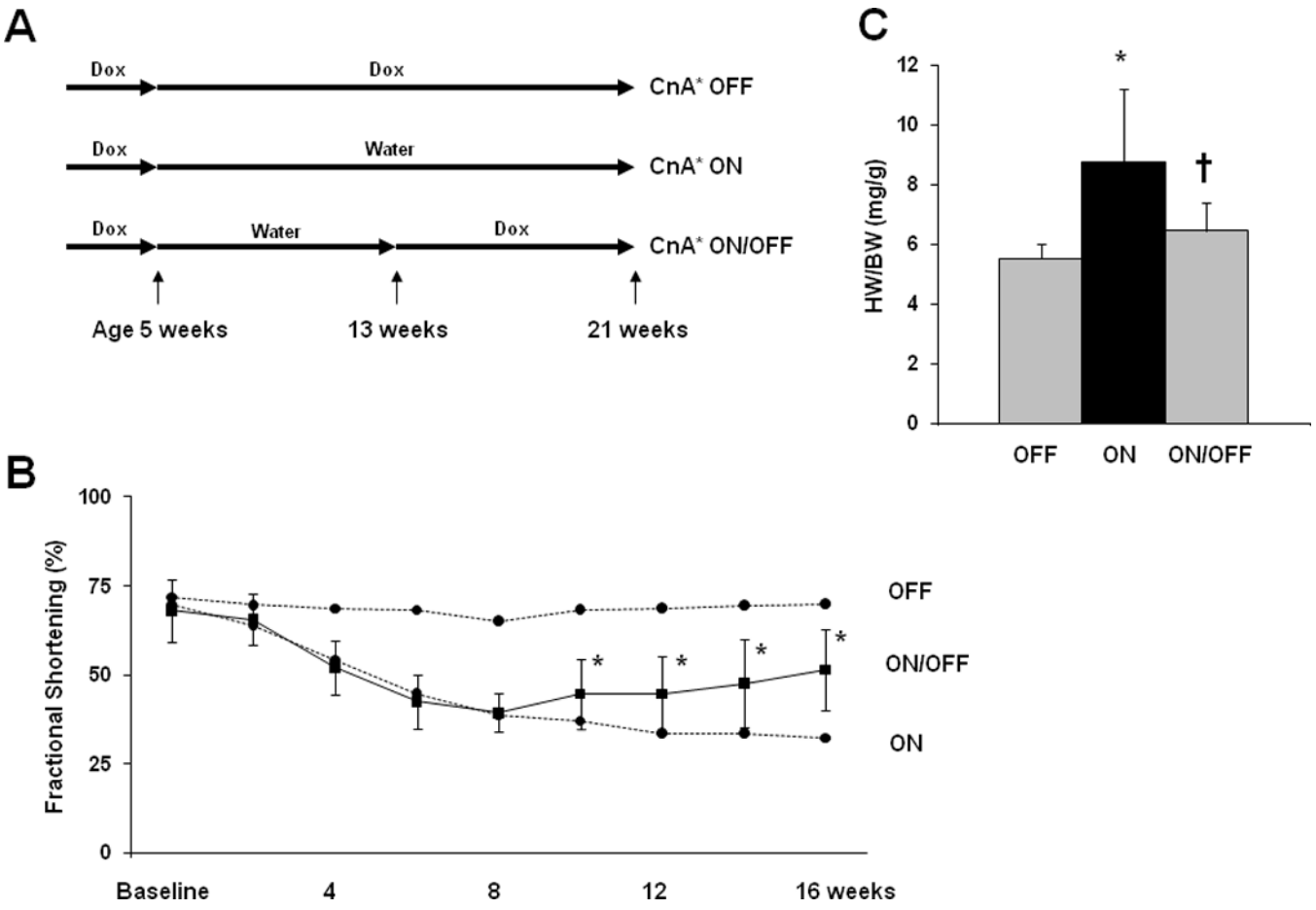
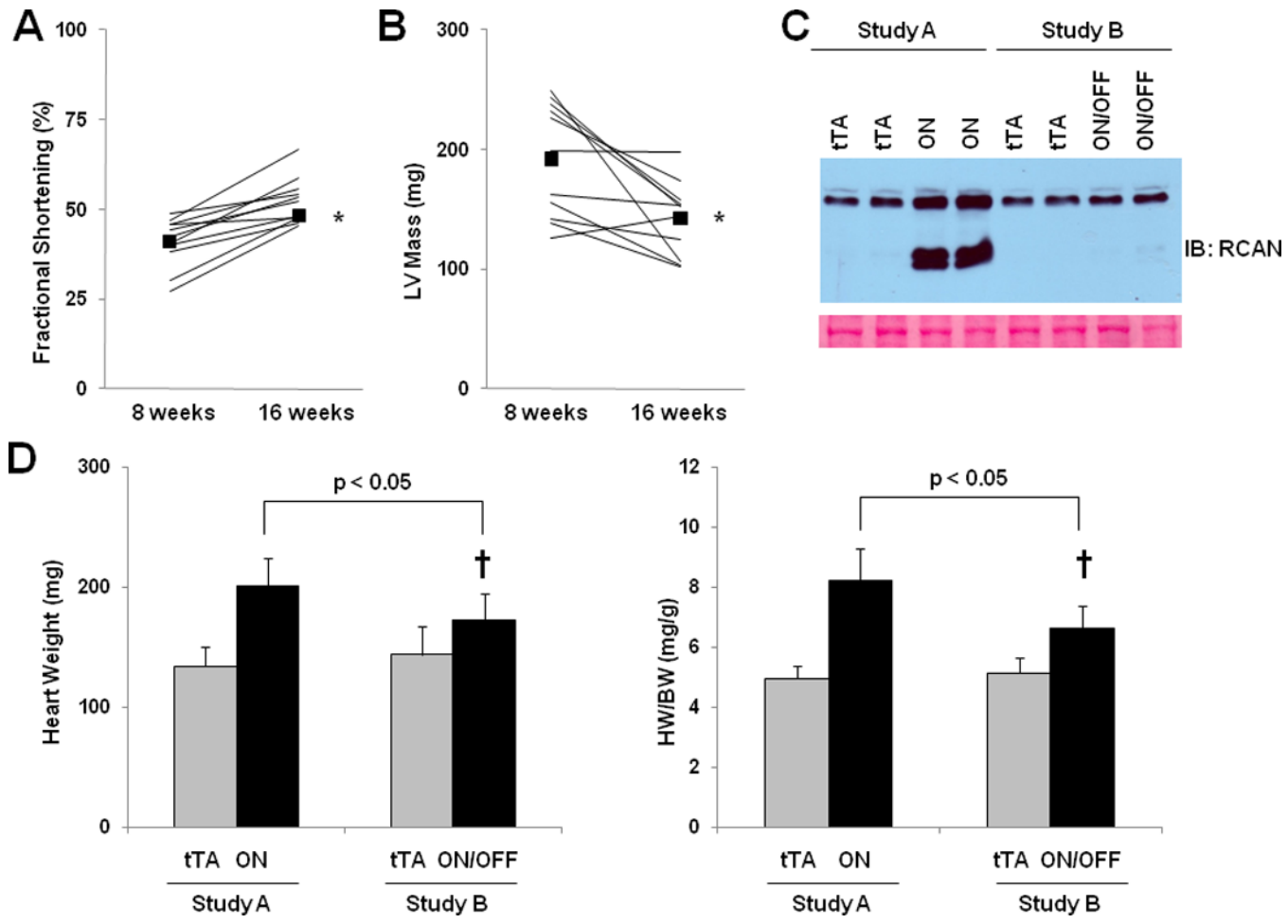


Figure 5. Impaired cardiac function recovers after activated calcineurin is extinguished

A: Experimental design. Double transgenic mice maintained on doxycycline were used as controls. B: Mean percent fractional shortening measured from serial M-mode tracings of tTA/CnA* mice in the ON/OFF group (n=16). Dotted lines represent mean percent fractional shortening in the OFF (n=10) and ON groups (n=14). *p < 0.01. C: Mean HW/BW at study completion. *p < 0.01 for ON vs OFF; † p < 0.01 for ON/OFF vs ON.



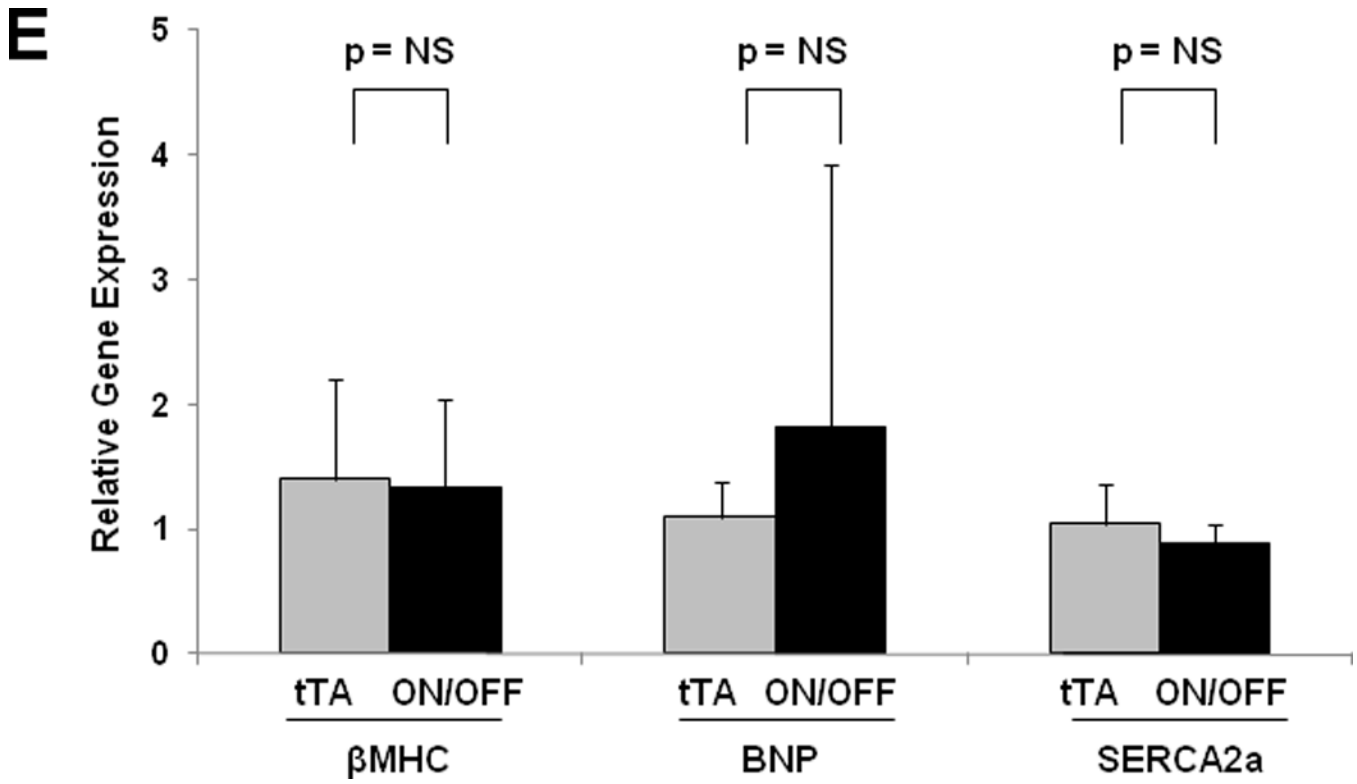


Figure 6. Pathological hypertrophy regresses after activated calcineurin is extinguished

Fractional shortening (A) and LV mass by echocardiography (B) for ON/OFF mice (n=11) before and after restarting doxycycline. Squares represent means. C: Representative immunoblot of LV lysates probed for RCAN from treatment groups as listed. Ponceau stain shows protein loading for each lane. D: Mean absolute heart weight and HW/BW for OFF (n=9) and ON (n=11) mice in study A and OFF (n=4) and ON/OFF (n=6) mice in study B. † $p < 0.05$ for ON/OFF (study B) vs. ON (study A) E: Mean mRNA levels of βMHC, BNP, and SERCA2a determined by real-time RT-PCR from RNA isolated from OFF (gray bars; n=4) and ON/OFF (black bars, n=6) hearts. mRNA levels are calculated relative to cyclophilin mRNA and normalized to control levels. F: Mean mRNA levels of MuRF3 determined by real-time RT-PCR from RNA isolated from OFF, ON, and ON/OFF hearts at 8 weeks (after removing doxycycline) or 16 weeks (after removing and then restarting doxycycline). ON: tTA/CnA* mice with Dox removed for 8 weeks; ON/OFF: tTA/CnA* mice with Dox removed for 8 weeks then restarted for 8 weeks; OFF: single transgenic tTA mice served as controls.