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Critical Roles of STAT3 in β-Adrenergic Functions in the Heart

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Abstract

Background—β-adrenergic receptors (βARs) play paradoxical roles in the heart. On one hand, βARs augment cardiac performance to fulfill the physiological demands, but on the other hand, prolonged activations of βARs exert deleterious effects that result in heart failure. The signal transducer and activator of transcription 3 (STAT3) plays a dynamic role in integrating multiple cytokine signaling pathways in a number of tissues. Altered activation of STAT3 has been observed in failing heart in both the human patients and animal models. Our objective is to determine the potential regulatory roles of STAT3 in cardiac βAR-mediated signaling and function.

Methods and Results—We observed that STAT3 can be directly activated in cardiomyocytes by β-adrenergic agonists. To follow up this finding, we analyzed βAR function in cardiomyocyte-restricted STAT3 knockouts and discovered that the conditional loss of STAT3 in cardiomyocytes

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markedly reduced the cardiac contractile response to acute βAR stimulation, and caused disengagement of calcium coupling and muscle contraction. Under chronic β-adrenergic stimulation, *Stat3cKO* hearts exhibited pronounced cardiomyocyte hypertrophy, cell death, and subsequent cardiac fibrosis. Biochemical and genetic data supported that Gαs and Src kinases are required for βAR-mediated activation of STAT3. Finally, we demonstrated that STAT3 transcriptionally regulates several key components of βAR pathway, including β_1AR and PKA, and T-type Ca^{2+} channels.

Conclusions—Our data demonstrates for the first time that STAT3 has a fundamental role in βAR signaling and functions in the heart. STAT3 serves as a critical transcriptional regulator for βAR-mediated cardiac stress adaption, pathological remodelling and heart failure.

Keywords

GPCR; βAR signaling; STAT3; heart failure

β-adrenergic receptors (βARs) belong to G protein-coupled receptor (GPCR) superfamily and are essential to cardiac physiology.[1, 2] The human heart mainly expresses β_1-adrenergic receptors (β_1AR) and β_2-adrenergic receptors (β_2AR) at a ratio of 7:3.[3] Under physiological conditions, βAR signaling regulates myocardial contraction and relaxation mainly through activation of Gαs-dependent mechanisms, such as the activation of adenylyl cyclase and protein kinase A (PKA).[4] βARs are closely associated with the progression of heart failure.[5] The hallmarks of heart failure include the specific down-regulation of β_1AR expression by up to 50%, uncoupling of β_1AR from Gαs, and an increase of Gαi coupling to antagonize Gαs signaling. However, the sustained activation of βAR/Gαs-dependent adenylyl cyclase signaling has also been suggested to exert deleterious effects on heart remodeling, leading to cardiac hypertrophy, cardiomyocyte death, and subsequent cardiac fibrosis, especially in failing hearts.[1, 6, 7] Therefore, regulation of the homeostasis of βAR signaling has a strong impact on both physiological and pathophysiological functions of the heart. In addition, βARs can exert their biological functions via signaling pathways independent of the second messenger cAMP-generating mechanisms.[8–10] Recent evidence has demonstrated that βARs are able to couple directly with other effectors and activate the ERK MAPK pathway, which has been suggested to promote the activation of a cardiac protective program to counteract the effect of catecholamine toxicity.[11, 12]

STAT (signal transducer and activator of transcription) is a family of transcriptional regulators that mediate a wide range of biological functions primarily in response to extracellular signaling molecules such as cytokines and growth factors.[13, 14] In general, STAT activation is through the phosphorylation of a single tyrosine residue that results in Src homology 2 (SH2) domain-mediated dimerization, nucleus localization, DNA binding, and ultimately transcription activation or repression. Recently, it has been also suggested that STAT3 (and probably other STATs as well) can exert its unique transcriptional activity without tyrosine phosphorylation.[15] STAT3 plays a convergent role in integrating multiple signaling pathways that are involved in a wide variety of physiological processes.[13, 14] Alterations in STAT3 activation and expression are associated with various pathophysiological adaptations in the heart, such as heart failures in humans and in a mouse model of dilated cardiomyopathy.[16–18] Cardiomyocyte-restricted ablation of STAT3

(*Stat3cKO*) in mice produces normal cardiac morphology and histology, suggesting that STAT3 is not critical to ventricular wall development, but develops cardiac hypertrophy and eventual heart failure in adult mice (> 6-month old).[19, 20] However, the underlying mechanism associated with this progressive heart failure has not been carefully characterized.

In this study, we have identified STAT3 as a key signaling element in βAR functions. STAT3 plays critical roles in regulating cardiac physiology via its transcriptional regulation of the homeostasis of βAR signaling components. By examining *Stat3cKO* hearts at young age, we discovered that the cardiac inotropic and lusitropic response to acute βAR stimulation was severely impaired. On the other hand, chronic βAR stimulation markedly enhanced hypertrophic response, cardiomyocyte death, and cardiac fibrosis in *Stat3cKO* mice. Biochemical analyses further demonstrated that STAT3 is an immediate signaling mediator of βARs, and that STAT3 acts as a master transcriptional regulator for several key signaling and functional components in βAR signaling pathway, including β_1AR, PKA, voltage gated L-type Ca^{2+} channel, and T-type Ca^{2+} channel subunits. Most importantly, STAT3 regulates these components under both basal and βAR activated conditions, and provides multi-phase control of cardiac physiology. This study reveals, for the first time, that STAT3 is a pivotal element of βAR signaling in the heart.

METHODS

Mice

Mice with deletion of STAT3 specifically in cardiomyocytes using MHC-cre (*Stat3cKO*) and their control littermates were maintained in C57 BL/6 genetic background.[19] Both *Stat3*$^{f/f}$ mice (*MHC-Cre* negative) and *Stat3*$^{f/+}$*; MHC-Cre*$^{+}$ mice were used as control mice, as they are undistinguishable in all aspects of histological and physiological measurements. MHC-Cre was kept as heterozygotes. For consistency, only 8 week-old young adult *Stat3cKO* male mice were used for experiments unless otherwise indicated. To effectively delete STAT3 in cardiomyocytes under inducible condition, *Stat3*$^{f/f}$ mouse were crossed with doxycycline-inducible *Tnnt2-rtTA;Tre-cre* mouse line (*Stat3icKO*) [21, 22]. The STAT3 deletion was thus carried out by feeding doxycycline-containing chow (Doxycycline 200mg/Kg, Bio-Serv) for 10 days. Cardiac hypertrophy induction by isoproterenol (ISO) was performed as previously described.[23] All animal protocols were approved by Fuwai Hospital and Indiana University School of Medicine Institutional Animal Care and Research Advisory Committee.

Echocardiography

Mice were lightly anesthetized with 1.5% isoflurane until the heart rate stabilized at 400 to 500 beats per minute. Two-dimensional short-axis images were obtained with a high resolution Micro-Ultrasound system (Vevo770, VisualSonics Inc, Toronto, Canada) equipped with a 40-MHz mechanical scan probe as previously described. [24]

Single cell calcium imaging

Myocytes were loaded with Fluo-4 AM (5μM, 20 min.) at room temperature. After de-esterification, the cells were perfused with normal Tyrode solution (1.8 mM Ca^{2+}) at 36°C ± 0.5. Confocal Ca^{2+} imaging was performed with a laser scanning confocal microscope (LSM 510 Meta, Carl Zeiss) equipped with a NA=1.35, 63x lens. Line scan measurements of Ca^{2+} transients, SR Ca^{2+} content and Ca^{2+} sparks were acquired at a sampling rate of 1.93 ms/line along the longitudinal axis of the myocytes. Ca^{2+} Sparks were measured under resting conditions. Steady state Ca^{2+} transients were achieved by a 30 sec pacing at 1 Hz. SR Ca^{2+} content was measured as Ca^{2+} release induced by 20 mM caffeine. All digital images were processed offline with IDL 6.0 (Research System Inc).

Isolated heart perfusion system (Langendorff)

Langendorff experiments were performed in isolated mouse hearts as described previously.[25, 26] Detailed protocol is described in supplemental materials.

Cell line

The MEF cells deficient of $G\alpha_s$ ($G\alpha_s^{-/-}$ cells) were derived from $G\alpha_s$ (exon 2) knockout mice.[27, 28] The MEF cells deficient in both β_1AR and β_2AR ($\beta_1^{-/-}\beta_2^{-/-}$ cells) were derived from β_1-AR and β_2-AR double knockout mouse embryos.[28] The MEF cells deficient in Src-family tyrosine kinases (SYF cells) were purchased from ATCC.

Statistical analysis

All values are presented as mean ± SEM. Statistical significance was determined by *t* test (for groups of two), or by one-way ANOVA t (for groups of three or more) using Sigma plot software package (Sigman). In the case that the results failed to pass the normality test, Wilcoxon Signed Rank T-test (for group of two), or Kruskal-Wallis methods for one-way ANOVA (for group three or more) were used to adjust the analysis and to determine the statistical significance. As the experiments analyzing calcium properties used multiple cells per animal, the experiment results were analyzed using a mixed-effect model with a “proc mixed” in the statistical software SAS 9.4 (details see: Supplemental materials and methods).

RESULTS

STAT3 activation by βAR in cardiomyocytes

As βAR-initiated signaling played critical roles in cardiac hypertrophy and heart failure and altered STAT3 activation was observed in failing hearts,[16–18] we tested if βAR agonists could activate STAT3 in the hearts. 2-month old male mice were administrated with isoproterenol (ISO) by single injection [1 μg/g bodyweight, intrapretoneal (ip)] and the levels of activated STAT3 (pY705-STAT3) were assessed by immunohistochemical (IHC) analyses. Activated STAT3 were detected within 30 minutes of ISO-administration in a small portion of cardiomyocytes. By 3 hours, almost all ventricular cardiomyocytes are positive for nuclear localized pY705-STAT3 (Figure 1A). Consistent with this finding,

Western blot was able to detect pY705-STAT3 at around 1 hour after ISO-administration (Supplemental Figure 1A).

To exclude non-cell autonomous effect on STAT3 activation by βAR agonist, we isolated cardiomyocytes from wild-type adult mice and stimulated with dobutamine (1 μM) in vitro. Dobutamine treatment could rapidly induce nuclear localization of STAT3 (Figure 1B) and pY705-STAT3 (Figure 1C) in cardiomyocytes within 15 minutes, demonstrating that STAT3, in addition to its well-defined roles in cytokine receptor signaling, is likely a down-stream signaling component of βARs.

βAR-initiated STAT3 activation requires $G\alpha_S$ and Src kinase

To investigate the molecular mechanism underlying βAR-mediated STAT3 activation, we took the advantage of the availability of established mouse embryonic fibroblast (MEF) cell lines with deletions of β_1AR/β_2AR, $G\alpha_s$, or Src-family tyrosine kinases, respectively. Oncostatin M (OSM), a cytokine of the IL-6 family that activates STAT3 via dimerization of the IL-6 receptor and Gp130, was used as a positive control. Figure 2A demonstrate that ISO-induced STAT3 activation is completely abolished in β_1AR/β_2AR deficient ($\beta_1AR^{-/-}/\beta_2AR^{-/-}$) cells, while STAT3 activation by OSM treatment is not affected, indicating that STAT3 activation by ISO is a βAR-dependent event. Furthermore, ISO-induced STAT3 activation was similarly abolished in Gαs-deficient MEF cells (Figure 2A), suggesting $G\alpha_S$-dependence for STAT3 activation by βARs. Consistently, while specific β_1AR antagonists (β-blockers) (metoprolol and bisoprolol) and specific β_2AR antagonist (ICI-181,551) failed to block ISO-induced STAT3 activation in neonatal mouse cardiomyocytes (NMCs) (Figure 2B and supplemental Figure 1D), the non-selective βAR antagonists (propranolol and carvedilol) completely abolished βAR-mediated STAT3 activation in NMCs (Figure 2B and supplemental Figure 1D).

Src-family protein tyrosine kinases are non-receptor kinases and have been known as major mediators for cytokine and various extracellular stimuli-induced STAT3 activation. Src-family kinases are known to be activated by βARs in $G\alpha_S$-dependent or -independent manners, and promote the activation of a second wave of signaling cascades including the ERK MAPK pathway.[8, 10] To test whether Src kinases were involved in βAR-initiated STAT3 activation, we used SYF cells which were $Src^{-/-}/Yes^{-/-}/Fyn^{-/-}$ MEF cells (Src, Yes and Fyn are the main Src-family kinases in MEF cells). As shown in Figure 2A, ISO-initiated STAT3 activation was abolished in SYF cells. These data suggest that βAR activation of STAT3 depends upon Src-family tyrosine kinases in MEF cells. To extend these observations, we examined STAT3 activation in cardiomyocytes treated with dobutamine with or without pre-incubation of Src kinase inhibitor I. Both Western blot and immunofluorescence staining confirmed that Src kinase inhibitor I inhibited βAR-initiated STAT3 phosphorylation (Figure 2C and 2E).

As several studies indicated that β_1AR is capable of trans-activating epidermal growth factor receptor (EGFR) to elevate MAPK signaling via a β-arrestin dependent pathway,[11, 12] we tested if STAT3 activation induced by βAR was also in part dependent upon the activation of EGFR. We pre-incubated neonatal cardiomyocytes with EGFR inhibitor AG1478. This treatment completely blocked the dobutamine-induced ERK activation, but had no effect on

dobutamine-induced STAT3 activation (Figure 2D). Collectively, our data demonstrate that βAR-mediated STAT3 activation is via Gαs and Src kinase.

Impaired βAR induced cardiac contractile function in *Stat3cKO* hearts

Next, we asked whether *Stat3cKO* hearts had an altered response to β-adrenergic stimulation. To exclude potential secondary effects from other exocrine systems on cardiac performance, we used the Langendorff system to directly measure and compare the contractile function of *Stat3cKO* and control hearts. As *Stat3cKO* mice did not have obvious altered cardiac morphology, histology and cardiac function under echocardiographic analysis before 2 months of age (Supplemental Figure 2), we used 2-month old hearts in the analyses. Of note, the treatment of β_1AR agonist dobutamine could dramatically stimulate LV contractile [dP/dt (max) and LV developed pressure – LVDP] and relaxation function [dP/dt (min)] in control hearts. However, we observed that this effect of dobutamine on LV function was markedly diminished in *Stat3cKO* hearts (Figure 3A–C), suggesting that STAT3 is critical for β-adrenergic signaling-initiated cardiac functions. As *Stat3cKO* hearts showed a comparable inotropic response to Na^+/K^+ ATPase inhibitor ouabain, indicating the inotropic reserve in STAT3 mutant hearts was at least partially maintained, and the impaired inotropic response to βAR agonists was unlikely due to the collapse of inotropic reserve in *Stat3cKO* hearts (Supplemental Figure 3A), As the phosphorylation of troponin I [p(S23/24)-troponin I] and the phosphorylation of the phospholamban [p(S16)-PLB] by PKA are two of the major events underlying β-adrenergic mediated signaling,[29] the levels of p-troponin I and p-PLB following 30 minutes of dobutamine stimulation were assessed by Western blots. We found that the base-line levels and the induction levels of p-troponin I and p-PLB were both significantly down-regulated in *Stat3cKO* hearts (Figure 3D). Consistently, up-regulation of second messenger cAMP induced by dobutamine was attenuated in *Stat3cKO* hearts when compared with controls (Figure 3E), suggesting that βAR-mediated signaling is impaired in *Stat3cKO* hearts. To further confirm that down-regulation of βARs/PKA signaling was not due to a secondary myocardium adaption resulted from an early stage ablation of STAT3 in myocardium, we used a doxycycline-inducible cre transgenic line (*Tnnt2-rtTA;Tre-cre*)[21] to ablate myocardial STAT3 in 2-month old mice (*Stat3icKO*). We found that similar to what we observed in *Stat3cKO* hearts in response to acute dobutamine treatment, the phosphorylation levels of troponin I [p(S23/24)] and PLB [p(S16)] were both reduced in the hearts of doxycycline-administered *Stat3icKO* mice when compared to *Stat3$^{f/f}$;Tnnt2-rtTA;Tre-cre* mice without doxycycline treatment (Supplemental Figure 3D). Taking together, this series of work demonstrate that STAT3 plays a key role in βAR signaling. Furthermore, as intracellular Ca^{2+} handling plays a central role in coordinating cardiac contraction and relaxation in response to β-adrenergic activation,[5] we measured the steady Ca^{2+} transient dynamics, SR Ca^{2+} content, and Ca^{2+} sparks in response to β-adrenergic stimulation in single cardiomyocytes isolated from control and *Stat3cKO* ventricles. At quiescent state, the Ca^{2+} transient amplitude and activation kinetics (T_{peak}) were comparable in control and *Stat3cKO* cardiomyocytes (Figure 3F and Supplemental Figure 4). Dobutamine stimulation significantly elevated the amplitude and accelerated the upstroke kinetics (T_{peak}) of Ca^{2+} transients in control cardiomyocytes, but not, or to a significantly lesser degree, in Stat3-deficient cardiomyocytes (Figure 3G). Similarly, the increases of SR Ca^{2+} content and the frequency of Ca^{2+} sparks in *Stat3cKO*

cardiomyocytes induced by dobutamine were diminished when compared with control cardiomyocytes (Figure 3H and I). Together, these data clearly demonstrate a critical role for STAT3 in βAR-mediated physiological function in cardiomyocyte.

Enhanced cardiomyocyte death in response to chronic β-adrenergic stimulation in *Stat3cKO* hearts

To investigate the function of STAT3 in response to chronic β-adrenergic stimulation, *Stat3cKO* mice and their control littermates were subjected to a 7-day isoproterenol administration using mini-osmotic pumps (1μg/g bodyweight/hour, 7 days). Cardiac function was assessed by echocardiographic analysis prior to and after ISO treatment. The 7-day β-adrenergic stimulation significantly enhanced cardiac systolic function, but simultaneously caused cardiac hypertrophy, cardiomyocyte death and cardiac fibrosis. As shown in Figure 4A, *Stat3cKO* mice showed an appreciable increase of cardiac contractile function in response to the chronic β-adrenergic stimulation, but the response was significantly attenuated when compared with that of littermate controls [FS (%): control mice, −ISO versus ISO: 40.18±4.1 to 52.6±3.1, n = 10; *Stat3cKO* mice, −ISO versus ISO: 40.54±3.7 to 47.7±2.5, n = 7)] (also see Supplemental Figure 5). Importantly, *Stat3cKO* mice developed a much more severe cardiac hypertrophy when compared with hearts from control littermates as demonstrated by the heart size, heart weight (normalized by tibia length) (Figure 4B), and cardiomyocyte transverse area (Figure 4C). Excessive cardiomyocyte necrosis and apoptosis were found in *Stat3cKO* hearts as compared to controls (Figure 5A and Supplemental Figure 6) which were accompanied by an increased level of perivascular and interstitial collagen deposition (Figure 5B). Consistent with the enhanced levels of cell death and fibrosis in *Stat3cKO* hearts, Western blot analyses demonstrated a significant reduction of Bcl-xl and an increase of TGFβ-signaling (*i.e.*, an increased level of pSmad2) in *Stat3cKO* hearts after 4 days of continuous ISO stimulation when compared with controls (Figure 5C). Additionally, we found that deletion of STAT3 in cardiomyocytes greatly increased STAT3 activation in vasculature and cardiac fibroblast cells under prolonged ISO stimulation (Figure 5D and E), which strongly suggests the role of STAT3 in regulating collagen synthesis in myofibroblast cells. The protective function of STAT3 in cardiomyocyte against chronic ISO stimulation was further confirmed in the doxycycline treated *Stat3icKO* hearts; there were increased levels of cardiomyocyte necrosis and myocardial fibrosis in in the doxycycline treated *Stat3icKO* hearts after prolonged ISO administration (Supplemental Figure 7).

βAR signaling components are direct transcriptional target of STAT3

To further understand the molecular mechanisms by which STAT3 contributes to the functions of βAR, we investigated the effect of STAT3 deficiency on the transcription of signaling components downstream of βAR. Previously, using genome-wide chromatin immunoprecipitation (ChIP) and gene expression array analyses, the occupancy of STAT3 on different chromatin regions was examined in embryonic stem cells.[30, 31] By exploring the published database in these studies, we found that the promoter regions of a number of key βAR signaling components (e.g., Adrb2, Ryr2, Serca2 and Cacna2d2, etc.) are occupied by STAT3 (Supplemental Table 1). To further investigate the transcriptional regulation in the heart, we surveyed the mRNA levels of major βAR signaling components in *Stat3cKO*

hearts, which included β1AR (Adrb1), β_2AR (Adrb2), PKA subunits, adenylyl cyclase (ACs), G protein-coupled receptor kinases (GRK2 and GRK5), and β-arrestin 2, and important effectors of βAR signaling in the hearts [*e.g.*, cardiac troponin I (TnI), ryanodine receptor 2 (Ryr2) and phospholamban (Pln), voltage-gated Ca^{2+} channel subunits]. As shown in Figure 6A, Adrb1, PKA catalytic subunit α and β (Prkaca and Prkacb), L-type calcium channel subunits Cacnα1c, and Serca2 were significantly down-regulated in *Stat3cKO* hearts. These genes are known to be critical to the cardiac inotrophic and lusitropic response. Down-regulation of these genes explains the phenotype of decreased responses to acute βAR stimulation in *Stat3cKO* hearts. GRKs and β-arrestin 2 expression were not found altered. Interestingly, the pore forming α-subunit of voltage-gated T-type Ca^{2+} channel (Cacnα1h) was significantly up-regulated in *Stat3cKO* hearts when compared with littermate controls (Figure 6A). Cacnα1h up-regulation has been implicated to be a contributing factor in cardiac hypertrophy and heart failure.[32–34] To understand whether these genes were direct targets of STAT3, we analyzed promoter regions of two critical signaling components, Adrb1 and Prkaca, and identified highly conserved STAT3 binding sites in the mouse Adrb1 and Prkaca promoter regions, located at nt-1602 and nt-5917 respectively (Figure 6B and C). ChIP assays demonstrated that STAT3 bound to both sites. Importantly, the binding activities increased dramatically in response to ISO treatment (Figure 6B and C). Additional experiments using luciferase assay further confirmed the function of both sites in response to STAT3 expression (Supplemental Figure 8).

Three potential STAT3 binding sites (S1, nt-4488; S2, nt-1309; S3, nt-1968) were initially identified in the promoter region of the mouse Cacnα1h gene by bioinformatics analysis (Figure 6D). ChIP analysis demonstrated that nt-1309 was the only binding site for STAT3 (Figure 6E). ISO-treatment enhanced the binding activity of STAT3 at nt-1309 site (Figure 6F). Unlike Adrb1 and Prkaca, ISO treatment suppressed Cacnα1h expression in control hearts, but not in *Stat3cKO* hearts (Figure 6G), further indicating that STAT3 is a negative transcriptional regulator of Cacnα1h.

Altered expression of voltage-gated T-type Ca^{2+} channel contributes to excessive cardiomyocyte death in *Stat3cKO* hearts

Given that calcium dynamics plays an important role in βAR mediated cardiac remodeling,[5] we are particularly interested in the altered expression levels of Cacnα1h in *Stat3cKO* hearts. Consistent with quantitative RT-PCR data, Western blot confirmed that Cacnα1c was down-regulated and Cacnα1h was significantly up-regulated in *Stat3cKO* hearts (Figure 7A), which suggested a reduced L-type calcium current and increased t-type calcium current. As a number of recent studies demonstrated that the increase of T-type calcium currents is closely associated with the adverse cardiac remodeling,[35, 36] we tested whether inhibition of T-type channel function will alleviate the cardiac hypertrophy and/or cardiomyocyte death and cardiac fibrosis in *Stat3cKO* hearts under chronic ISO stimulation. A T-type Ca^{2+} current suppressing does of efonidipine (25mg/kg bodyweight)[33] was given to *Stat3cKO* mice (2-month old) under 7-day isoproterenol administration. We found that efonidipine treatment did not suppress chronic β-adrenergic stimulation induced cardiomyocyte hypertrophy; the heart weight-to-tibia length ratio and cardiomyocyte transverse area (μ^2) were comparable between the mice treated with either isoproterenol

alone or co-treated with efonidipine (Figure 7B). However, there was a significant reduction of cardiomyocyte death and cardiac fibrosis induced by β-adrenergic stimulation when efonidipine was co-administrated in *Stat3cKO mice* (Figure 7C and D). This data revealed that the elevated level of T-type calcium current is likely one of the major contributing factors to β-adrenergic-mediated cardiomyocyte death.

DISCUSSION

βAR signaling is essential for cardiac function under both physiological and pathophysiological conditions. As one of the most important targets of cardiovascular therapeutic drugs, understanding the mechanism by which the βAR signaling pathway is regulated has been a major effort in biomedical research in the past two decades. However, due to the complexity of its associated pathways, one key question- how βAR signaling elements are transcriptionally regulated- remains largely unanswered. Our current study demonstrates, for the first time, that STAT3 acts as a converging transcriptional regulator of several critical signaling components to maintain the normal homeostasis of βAR signaling. This function of STAT3 is essential for normal βAR-mediated cardiac contractile physiology and suppressing adverse myocardial remodeling (Figure 8); the latter is highly relevant to the pathogenesis of heart failure.

Our study demonstrates that STAT3 function is one of the non-second messenger-generating signaling pathways activated by βARs in cardiomyocytes. The notion that βARs could trigger STST3 activation had been previously noted in non-cardiac cell types (e.g., myofibroblast cells). However, this was attributed to a secondary non-cell autonomous event of induced expression of cytokine IL-6.[37, 38] Here we have demonstrated that βAR-mediated STAT3 activation is via a direct mechanism, and depends upon β_1AR and β_2AR, Gαs and Src-family kinases. Furthermore, while Src-family kinases are involved in both STAT3 and ERK activation, βAR-mediated trans-activation of EGFR is only required for ERK, but not STAT3 activation. In addition, deletion of Gαs in MEF cells completely abolishes STAT3 activation by βAR even at higher dose of ISO treatment (> 1 μM), which is different from βAR-mediated ERK activation that only requires Gαs at lower dose (<0.1μM).[28] These data suggest that there exist distinct signaling transduction cascades for the activation of different non-second messenger-generating signaling pathways. A growing body of evidence suggests that βARs activate additional signaling factors in parallel to second messenger-generating mechanisms to exert their biological functions.[8–10] Rockman and his colleagues have demonstrated that βAR-mediated ERK activation, via an EGFR and β–arrestin dependent mechanism, promotes the cardiac protective program and counteracts the effect of catecholamine toxicity.[11, 12] ERK activation in *Stat3cKO* mice is normal in both basal and β-AR agonist stimulated states (Figure 5), suggesting that STAT3 does not exert its cardiac protective activity via ERK.

The activation of STAT3 provides another cardiac protective mechanism. The analyses of *Adrb1* and *Pkacb* expression, and STAT3 binding to their promoter regions demonstrate that STAT3 is a direct positive transcriptional regulator of *Adrb1* and *Pkacb*. Previously, it was shown that STAT3 occupied the promoter region of a number of key βAR signaling components (Supplemental Table 1 for a list of genes).[30, 31] All these genes are known to be

critical components in βAR signaling. Thus, STAT3 is primarily responsible for maintaining the homeostasis of βAR signaling. In the absence of STAT3, hearts become insensitive to acute β-adrenergic stimulation due to the down-regulation of β1AR, PKA, and several other downstream effectors (Figure 8).

In addition to the reduced sensitivity to acute β-adrenergic stimulation in *Stat3cKO* hearts, we observed increased cell death and cardiac fibrosis in response to chronic β-adrenergic stimulation. We attribute this phenomenon partially to the fact that STAT3 is a negative regulator of T-type Ca^{2+} channels (Cacnα1h) (Figure 7 and Figure 8) and a positive regulator of pro-survival factor Bcl-xl.[39] The T-type calcium channel subunit, Cacnα1h, is dramatically increased in *Stat3cKO* hearts when compared to controls (Figure 7). Normally, Cacnα1h is abundantly expressed in embryonic hearts and subsequently down-regulated in the adult ventricular cardiomyocyte, while remaining high in atrial cardiomyocytes and cardiac conductive cells.[36] It has been shown that the recurrence of inward Ca^{2+} T-currents ($I_{Ca,\ T}$) in adult ventricular cardiomyocytes is a contributing factor in the progression of heart failure.[40] Administration of Ca^{2+} channel blocker, efonidipine, significantly blocked cell death and heart fibrosis induced by βAR-agonist in *Stat3cKO* mouse. Considering that there is a decrease of L-type calcium channel subunit Cacnα1c protein expression in *Stat3cKO* hearts, suggesting a reduced L-type calcium channel function, the reduction of cardiommyocytes death mediated by efonidipine administration under chronic ISO stimulation is most likely due to the inhibition of elevated T-type calcium channel function. This finding further suggested the contribution of elevated $I_{Ca,\ T}$ to the heart failure phenotype in *Stat3cKO* mice. However, as efonidipine can also inhibit L-type Ca^{2+} channel at higher dose,[41] we cannot fully exclude partial contribution from its inhibitory activity to L-type channel. Therefore, the future study using mice with genetically modified T-type and L-type Ca^{2+} channel subunits are necessary to further validate the role of both types of Ca^{2+} channels in mediating the cardiomyocytes intolerance to catecholamine stress in *Stat3cKO* mice. One possibility is that the elevated $I_{Ca,\ T}$ exacerbates heart failure via the activation of NFAT,[35] as conditional ablation of Cacnα1h leads to diminished NFAT activation in response to transverse aortic constriction (TAC). In *Stat3cKO* hearts, there is a significant increase of nuclear NFATc1 in cardiomyocytes (Supplemental Figure 9). Therefore, another potentially important function for STAT3 is to prevent over-activation of Cacnα1h in the heart. Although the genetic model used in this study specifically ablates STAT3 in cardiomyocytes, it leads to a great enhancement of STAT3 activation in vascular and interstitial myofibroblasts (Figure 5D and E) under prolonged ISO stimulation. This phenomenon may suggest a critical role of STAT3 in the cellular cross-talk between cardiomyocytes and other cell types in the heart, possibly via a paracrine pathway during inflammation or in response to oxidative stress in injured myocardium.[16] The hyperactivation of STAT3 noted in myofibroblasts also reflects the complexity of STAT3 in different cardiac cell types, which could contribute directly at different pathogenic phases in diseased and failing hearts to develop excessive reparative and reactive fibrosis. Interestingly, a previous report demonstrated that hyperactivation of STAT3 led to desensitize TGFβ signaling.[42] Consistent with that, we found that prolonged ISO treatment leads to elevated TGFβ-signaling (*i.e.*, an increased level of pSmad2) in *Stat3cKO* hearts. Although the exacerbated oxidative stress induced by STAT3 deletion could be a major

factor,[43] the exact underlying mechanisms for the elevated TGFβ signaling in *Stat3cKO* hearts is still elusive and worth to be further studied.

One of the hallmarks of heart failure is the reduction of β_1AR and cardiac insensitivity to β-adrenergic stimulation.[44, 45] Given our finding that STAT3 directly regulates β_1AR expression, this might provide a molecular basis for the observed heart failure in older *Stat3cKO* mice. It is known that STAT3 activation is altered in various human heart failure patients.[17, 46] Based on our study, we propose a pivotal role for STAT3 in preventing the pathogenesis of heart failure in humans. Furthermore, in a mouse model carrying double mutations in cardiac Troponin I and α-myosin heavy chain (*tni-203/mhc-403*), which mimics a familial hypertrophic cardiomyopathy in humans,[47] STAT3 has been found to be activated in cardiomyocytes during the course of heart failure.[18] It is reasonable to propose that, based on our data, this activation of STAT3 provides a cardiac protective mechanism for the diseased heart. It would be interesting to generate compound mutant mice with a reduced level of STAT3 in *tni-203/mhc-403*, and analyze the impact of STAT3 in the development of hypertrophic cardiomyopathy and heart failure in future experiments.

In summary, we have shown that, in the myocardial-restricted STAT3 knockout mice, cardiac contractile function and calcium release properties by acute βAR stimulation are impaired. Furthermore, chronic βAR stimulation induces excessive hypertrophy, myocyte death and cardiac fibrosis in the absence of STAT3. Moreover, we have revealed the molecular mechanisms by which STAT3 contributes to βAR functions. βARs activate STAT3 through Gαs and Src kinases, and STAT3 directly regulates the gene expression of several key components in the adrenergic signaling pathways. Together, these data clearly demonstrate critical roles for STAT3 in β-adrenergic functions in the heart.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Clinical Perspectives

βAR signaling plays a central role in cardiac stress adaption, pathological remodeling and heart failure. Diminished contractile responsiveness to βAR stimulation is often correlated with increase of myocyte apoptosis, compensatory hypertrophy and cardiac fibrosis in failing human heart. The STAT (signal transducer and activator of transcription) family of transcriptional regulators mediates a wide range of biological functions. Tyrosine phosphorylation in response to varies of cytokine and stress stimulation is required for STAT3 dimerization, nuclear translocation, and DNA binding. Previous studies demonstrated that STAT3 activation and expression was severely reduced in failing hearts. Here, we show that STAT3 plays a critical role in maintaining cardiac homeostasis via its transcriptional regulation of major βAR signaling components. Conditional loss of STAT3 in cardiomyocytes impairs the inotropic and lusitropic response to acute βAR stimulation, and augments chronic βAR stimulation-induced hypertrophic response, cardiomyocyte death, and cardiac fibrosis. These findings suggest a novel pharmacological intervention for the management for heart failure. In addition, pharmacologic blockage of STAT3 signaling has been explored for the treatment of several malignancies; the results presented here suggest that close monitoring of cardiac function in these patients during treatment is warranted.

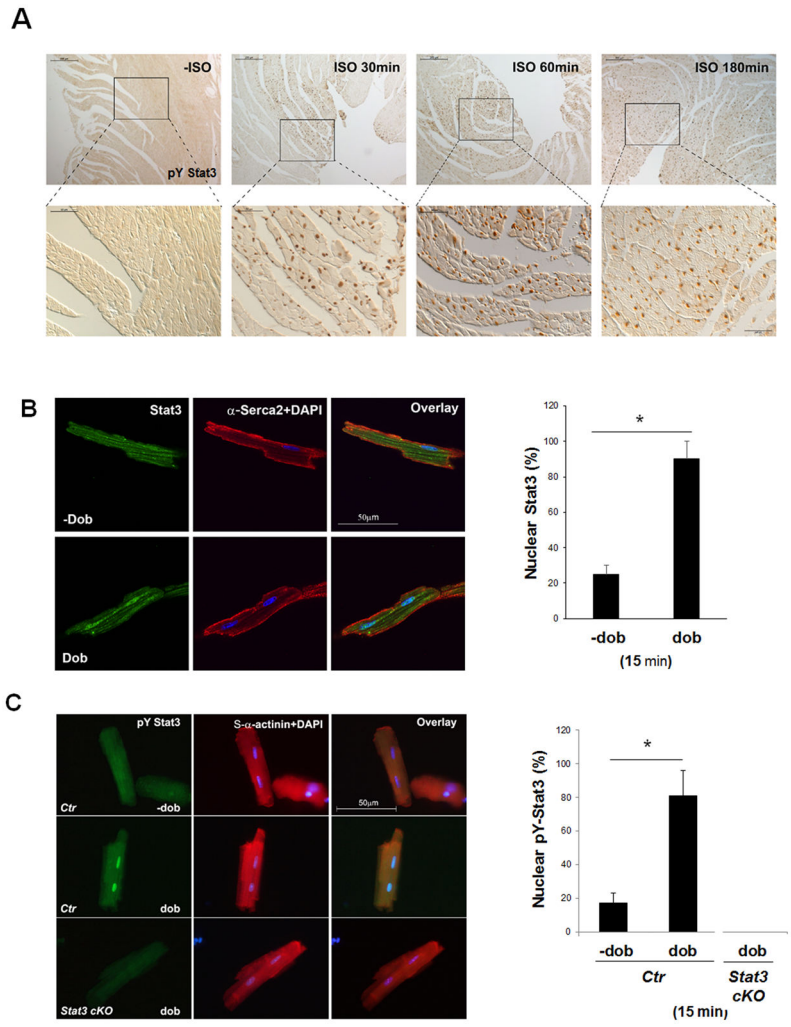

Figure 1.

Activation of STAT3 by βARs in cardiomyocytes. **A,** Representative images of immunohistochemical staining of pY-STAT3 in wild-type mouse hearts treated with ISO or saline (−ISO) for indicated amount of time via ip injection (1μg/g bodyweight.). **B,** Representative fluorescence images (left panel) and quantification (right panel) of nuclear STAT3 in isolated wild-type cardiomyocytes with or without dobutamine stimulation (dob) (1μM, for 15 minutes). Positive immune reactivity to anti-STAT3 antibody is shown in green and to anti-Serca2 antibody is shown in red. The experiments were repeated independently 3 times (N > 300 cells/each group counted). **C**, Representative fluorescence images (left panel) and quantification (right panel) of pY-STAT3 staining in isolated wild-type cardiomyocytes treated with or without dob (1μM, for 15 minutes). Positive immune reactivity to anti-pY-STAT3 antibody is shown in green and to anti-α–actinin (sarcomere) antibody is shown in red. *Stat3cKO* cardiomyocytes were used as a negative control for immune reactivity of the anti-pY-STAT3 antibody. The experiments were repeated independently 3 times (N > 300 cells/each group).

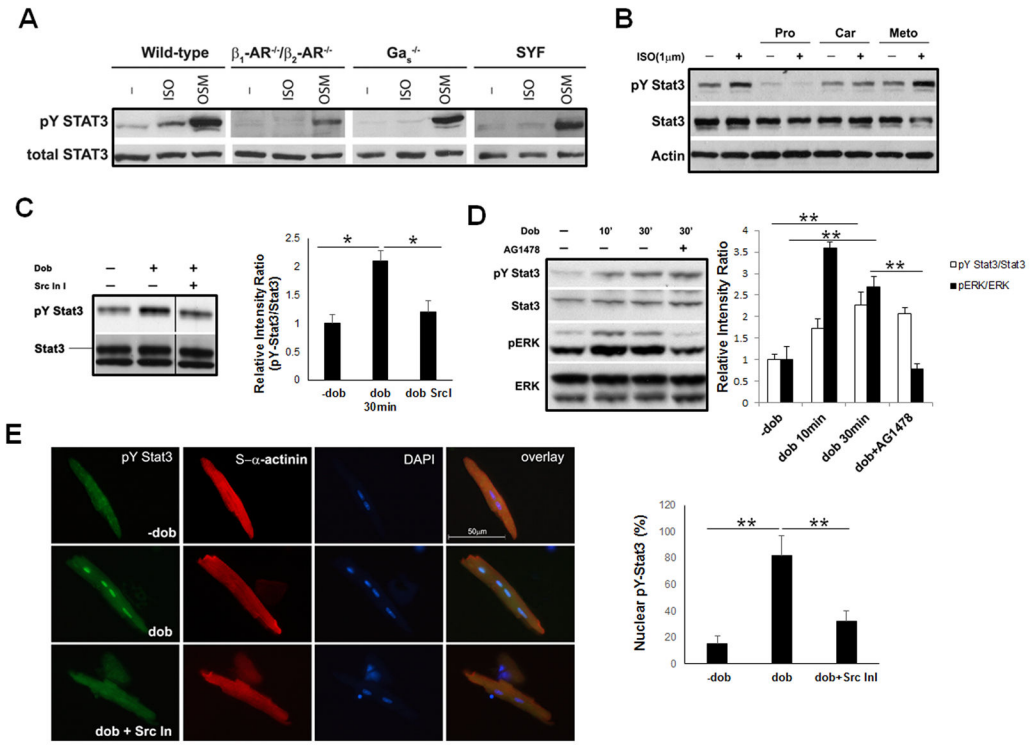

Figure 2.

Activation of STAT3 by βARs requires G α_s and Src-family kinases. **A,** Western blot analysis of STAT3 activation in response to ISO (1 μM, 30 minutes) in β_1AR/β_2AR-compound deficient, G α_s-deficient, and Src family kinases (SYF)-deficient mouse embryonic fibroblast (MEF) cells. OSM treatments were used as a positive control for pY-STAT3. **B,** Western blot analysis of STAT3 activation in neonatal cardiomyocytes in response to ISO (1 μM, 30 minutes) that pre-incubated with different βARs antagonists for 1 hour. Pro, propranolol; Car, carvedilol; Meto, metoprolol. **C**, Western blot analysis of pY-STAT3 in mouse neonatal cardiomyocytes with or without pre-incubation of Src kinase inhibitor I (1 μM) for 15 minutes, followed by dobutamine (1 μM) treatment. The relative intensity ratios between pY-STAT3 and total STAT3 are shown in the right panel, *P<0.05. **D,** Western blot analysis of pY-STAT3 and pERK activation in mouse neonatal cardiomyocytes with or without pre-incubation of EGFR inhibitor AG1478 (1 μM) followed by dobutamine (1 μM) treatment. The relative intensity ratios of pY-STAT3 vs total STAT3 and pERK1/2 vs total ERK1/2 are shown in right panel, **P<0.01 **E,** Representative fluorescence images of immune reactivity to anti-pY-STAT3 antibody (in green) in isolated wild-type cardiomyocytes treated with dobutamine (1 μM) for 30 minutes with or without pre-incubation of Src kinase inhibitor I (1 μM). α-actinin, in red; DAPI, in blue. The experiments were independently repeated 3 times (N >300 cells/each group) and results are expressed as positive cell number over total cell number (Right panel), **P<0.01.

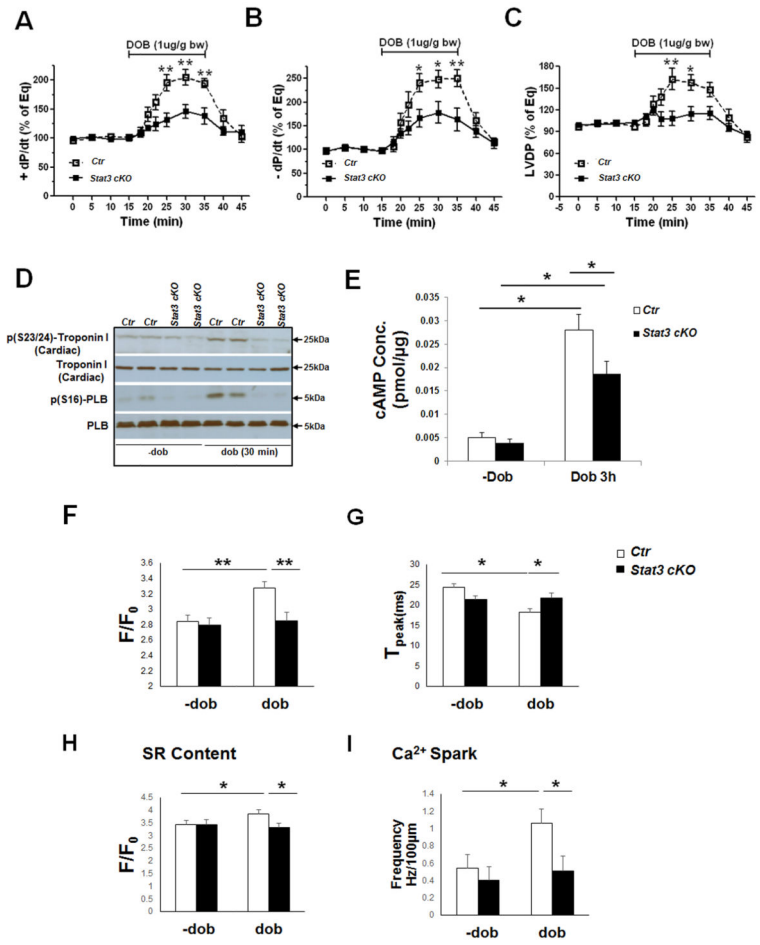

Figure 3.

Impaired βAR agonist-induced cardiac contractile function and blunted calcium coupling in STAT3-deficient hearts and cardiomyocytes. **A**–**C**, Evaluation of acute inotropic (**A**), lusitropic (**B**) responses, and LV developed pressure (LVDP) (**C**) in *Stat3cKO* and control hearts in response to dobutamine stimulation using a Langendorff perfusion system. Dobutamine was infused for 20 minutes. (bw: body weight ; Mean ± SEM; N=6/each group; *p<0.01 and **p<0.001). **D**, Western blots show that the levels of phosphorylated troponin I and phospholamban (PLB) are reduced in *Stat3cKO* hearts when compared to controls after 30 minutes of dobutamine stimulation. **E**, cAMP levels in *Stat3cKO* hearts are significantly lower compared to controls treated with dobutamine stimulation (*N* = 3/each group; *p<0.05). **F**–**G**, Comparison of steady-state Ca^{2+} transients from control and *Stat3cKO* cardiomyocytes. Prior to dobutamine treatment, Ca^{2+} transient amplitudes (F) and T_{peak} (activation kinetics) (G) are comparable in control and *Stat3cKO* cardiomyocytes. (Control: N = 17 cells/4 mice; *Stat3cKO*: N = 17 cells/3 mice). Dobutamine stimulation increases the amplitude and speeds the kinetics (T_{peak}) of control cardiomyocytes. In contrast, the amplitude and T_{peak} of Ca^{2+} transient in *Stat3cKO* cells are not affected by dobutamine stimulation. **H**, Assessment of SR Ca^{2+} content of control and *Stat3cKO* ventricular cardiomyocytes. Prior to dobutamine treatment, SR Ca^{2+} content is similar between control and *Stat3cKO* ventricular myocytes. In response to dobutamine treatment, SR Ca^{2+} content

is increased in control, but not in *Stat3cKO* cardiomyocytes (Control: N = 15 cells/3 mice; *Stat3cKO*: N = 18 cells/3 mice). **I,** Measurement of spontaneous Ca^{2+} spark frequency in control and *Stat3cKO* ventricular cardiomyocytes. Prior to dobutamine treatment, Ca^{2+} spark frequency is comparable in control and *Stat3cKO* cardiomyocytes. In response to dobutamine treatment, Ca^{2+}spark frequency increases significantly in control cells, but not in *Stat3cKO* cells. (Control: N = 20 cells/3 mice; *Stat3cKO*: N = 21 cells/3 mice). *P<0.05, **P<0.01.

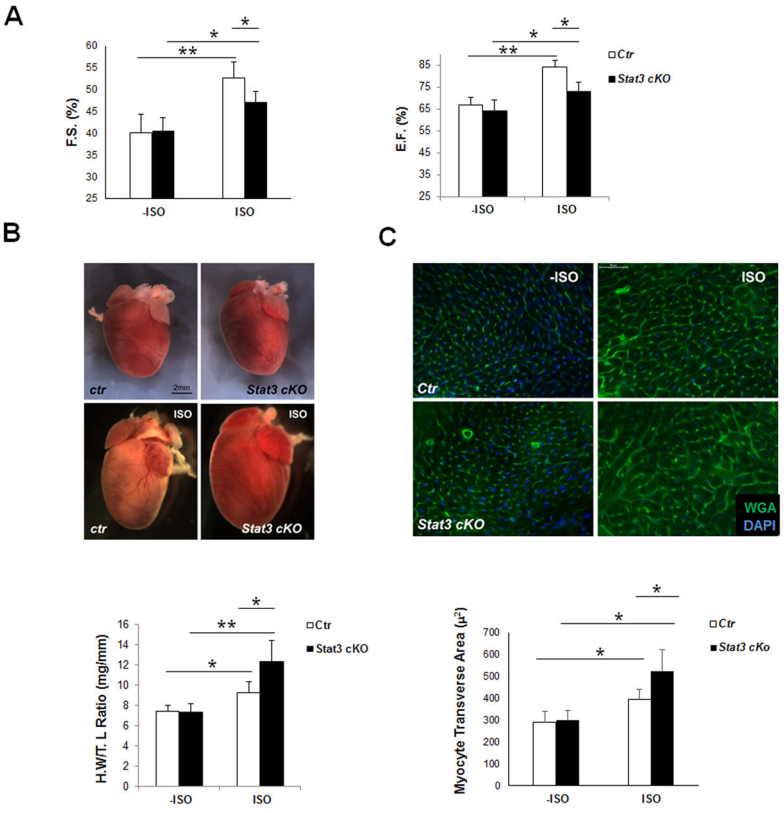

Figure 4.

Chronic administration of isoproterenol (*ISO*) induces excessive hypertrophy in *Stat3cKO* hearts. **A**, Echocardiographic analysis of cardiac function of control and *Stat3cKO* mouse (2-month old) in response to chronic ISO treatment. Left panel shows fractional shortening (FS%); Right panel shows ejection fraction (EF%) (Control: N = 10; *Stat3cKO*: N = 7), *P<0.05, **P<0.01. **B,** Upper panel shows representative images of control and *Stat3cKO* hearts (2-month old) prior to and after 7-day ISO perfusion. Lower panel shows heart weight vs tibia length ratios (N = 7/each group) of *Stat3cKO* hearts compared to controls after 7-day ISO perfusion, *P<0.05, **P<0.01. **C**, Upper panel shows representative fluorescent images of WGA (wheat germ agglutinin) staining (in green) of comparable left ventricular region of control and *Stat3cKO* hearts prior to and after 7-day ISO perfusion; DAPI, in blue. Lower panel shows the quantitation of cardiomyocyte transverse area (in μ^2) of *Stat3cKO* and control hearts after 7-day ISO perfusion (N = 5/each group), *P<0.05, **P<0.01.

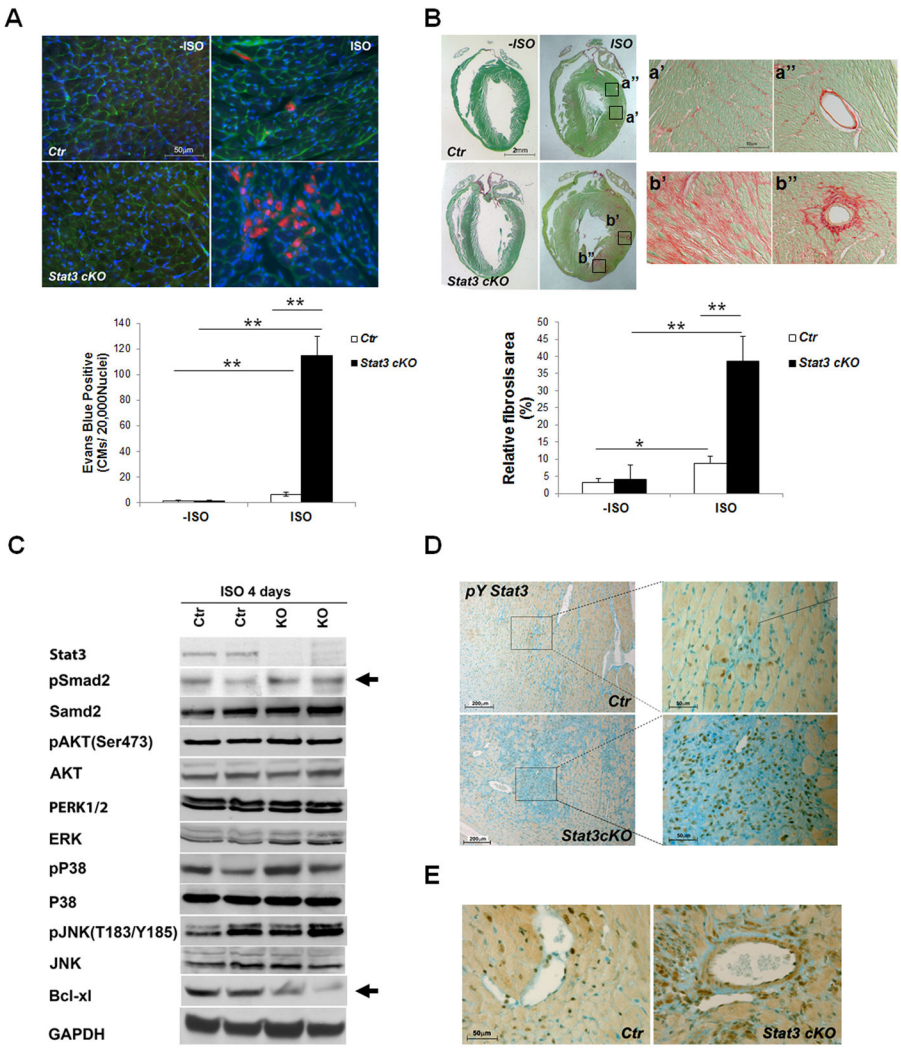

Figure 5.

Chronic administration of isoproterenol causes excessive myocyte death and cardiac fibrosis in *Stat3cKO* hearts. **A**, Chronic ISO stimulation induces excessive cardiomyocyte death in *Stat3cKO* hearts. Upper panel shows representative images of Evans blue (in red) uptake in control and *Stat3cKO* hearts with or without 7-day ISO perfusion (400x). WGA, in green; DAPI, in blue. Lower panel shows the quantitation of Evans blue positive myocytes in control and *Stat3cKO* hearts (N = 5/each group). **B,** Upper panel shows Sirius red and fast green staining to demonstrate the collagen deposition in control and *Stat3cKO* hearts with or without 7-day ISO perfusion (N = 5/each group). Lower panel shows the quantification of collagen contents, *P<0.05, **P<0.01. **C**, Representative results of Western blots show the reduced levels of Bcl-xl and the increased levels of pSmad2 in *Stat3cKO* hearts after 4 days of continuous ISO stimulation when compared with controls. **D**–**E,** Representative images of IHC staining of pY-STAT3 in control and *Stat3cKO* hearts after 7-day ISO perfusion. Activated STAT3 are found enhanced in myofibroblast cells in *Stat3cKO* hearts.

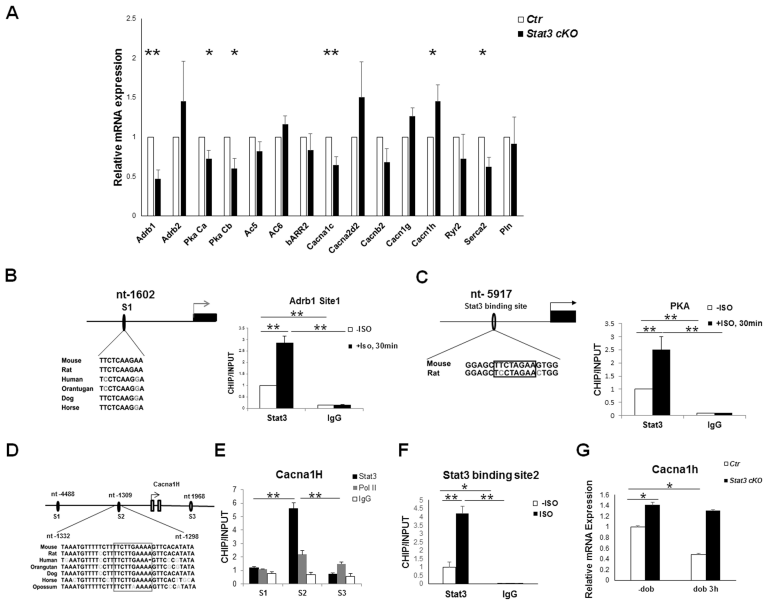

Figure 6.

βAR signaling components as direct targets of STAT3. **A,** Quantitative RT-PCR analyses of βAR signaling components and calcium handling genes from hearts of 2-month old control and *Stat3cKO* mice (N = 3/each group). Ribosomal protein L7 mRNA was used for normalization. **B**, Positive regulation of *Adrb1* by STAT3. Chromatin immunoprecipitation (ChIP) analysis of the STAT3 binding site in the promoter region of *Adrb1* at nt-1602 relative to the transcription start site (TSS). **C,** Positive regulation of *Prkaca* by STAT3. ChIP analysis of the STAT3 binding site in the promoter region of *Prkaca* at nt-5917 relative to its TSS. **D,** Schematic diagram of putative STAT3 binding sites at the promoter region of Cacna1h. **E–F,** ChIP assay in combination with quantitative PCR demonstrates the active STAT3 binding site and its response to ISO stimulation. **G,** Cacna1h expression in 2-month old control and *Stat3cKO* hearts with or without dobutamine treatment (1 μg/g bodyweight), *P<0.05, **P<0.01.

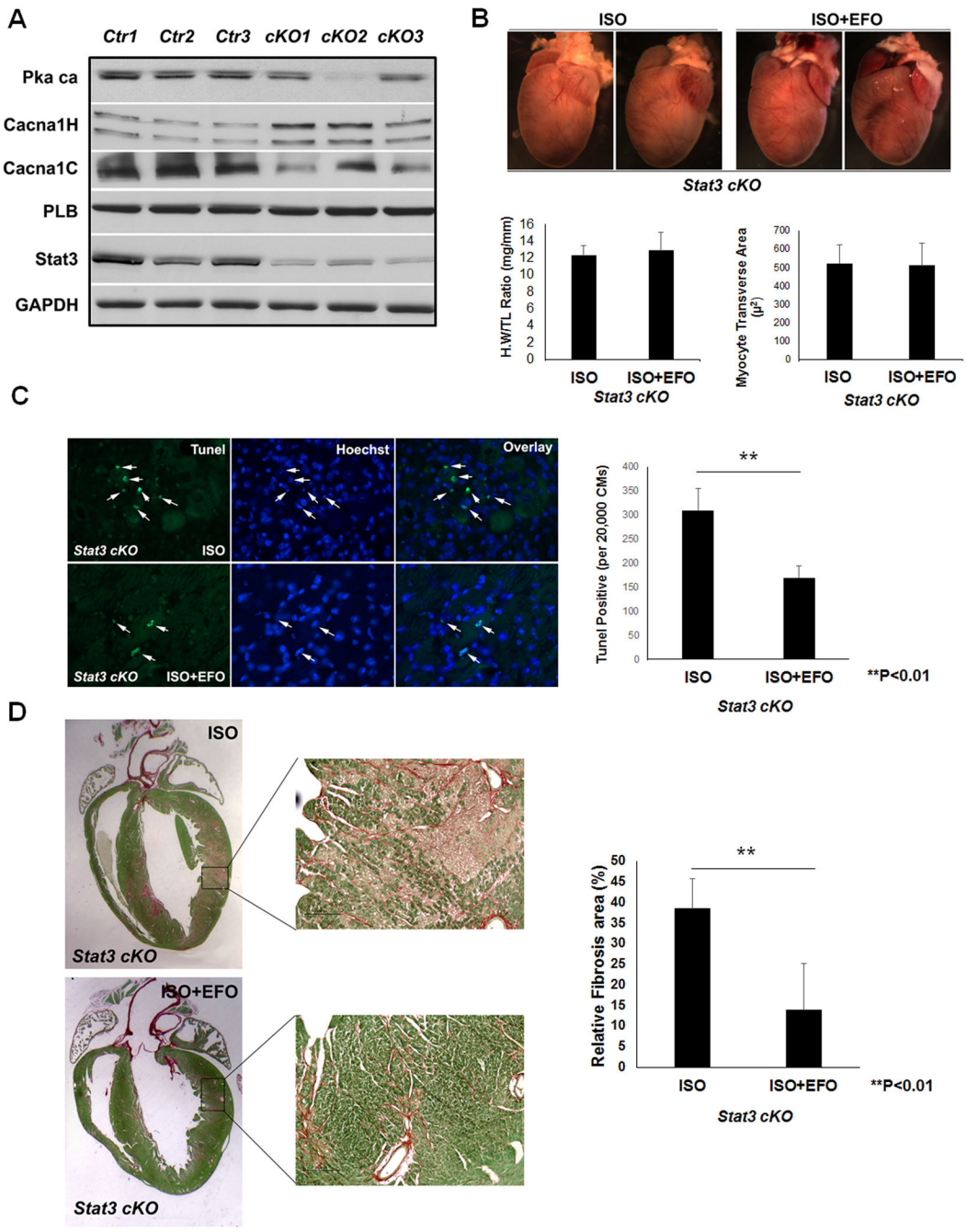

Figure 7.

Altered expression of Ca^{2+} channel protein contributes to excessive cardiomyocyte death in *Stat3cKO* hearts. **A,** Representative Western blot analysis of Pkaca, Cacna1H, Cacna1C, and PLB protein expression in control and *Stat3cKO* mouse heart. **B,** Administration of Efonidipine (EFO) did not affect the cardiomyocyte hypertrophy induced by chronic isoproterenol stimulation in *Stat3cKO* mouse hearts. Representative images of the hearts from *Stat3cKO* mice under chronic ISO stimulation alone (*Stat3cKO*, ISO) or in combination with oral administration of efonidipine (*Stat3cKO*, ISO+EFO). Heart weight vs

tibia length ratios is shown in left lower panel (*Stat3cKO*, ISO: N = 9; *Stat3cKO*, ISO+EFO: N = 6). Cardiomyocyte transverse area (μ^2) is shown in the right lower panel (*Stat3cKO*, ISO: N = 3; *Stat3cKO*, ISO+EFO: N = 3). **C,** Efonidipine administration alleviated cardiomyocytes death and cardiac fibrosis induced by chronic isoproterenol stimulation in *Stat3cKO* hearts. Representative images of TUNEL staining (green) of *Stat3cKO* hearts treated with ISO for 7 days alone or co-administrated with Efonidipine (400x). Right panel shows the quantitation of TUNEL positive myocytes in *Stat3cKO* ISO vs *Stat3cKO* ISO +EFO mouse heart (N = 3/each group, **P<0.01). **D,** Sirius red/fast green staining showing the collagen deposition (red signal) is markedly decreased in *Stat3cKO* heart with co-administration of Efonidipine in comparison with *Stat3cKO* heart treated with ISO alone for 7 days (N =3 in each group, **P<0.01).

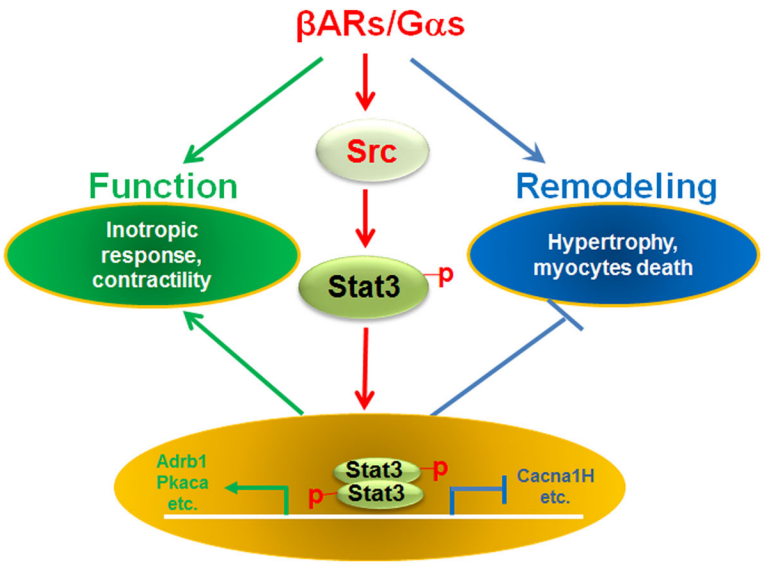

Figure 8.

Regulatory functions of STAT3 in cardiac β-adrenergic functions. Activation of STAT3 via the βAR-$G\alpha_s$/Src pathway has an important regulatory role in maintaining normal cardiac function and in minimizing adverse cardiac remodeling in response to β-adrenergic stimulation.