



RESEARCH PAPER

Stimulation of β -adrenoceptors up-regulates cardiac expression of galectin-3 and BIM through the Hippo signalling pathway

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Background and Purpose: Expression of the pro-fibrotic galectin-3 and the pro-apoptotic BIM is elevated in diseased heart or after β -adrenoceptor stimulation, but the underlying mechanisms are unclear. This question was addressed in the present study.

Experimental Approach: Wild-type mice and mice with cardiac transgenic expression of β_2 -adrenoceptors, mammalian sterile-20 like kinase 1 (Mst1) or dominant-negative Mst1, and non-specific galectin-3 knockout mice were used. Effects of the β -adrenoceptor agonist isoprenaline or β -adrenoceptor antagonists were studied. Rat cardiomyoblasts (H9c2) were used for mechanistic exploration. Biochemical assays were performed.

Key Results: Isoprenaline treatment up-regulated expression of galectin-3 and BIM, and this was inhibited by non-selective or selective β -adrenoceptor antagonists (by 60–70%). Cardiac expression of galectin-3 and BIM was increased in β_2 -adrenoceptor transgenic mice. Isoprenaline-induced up-regulation of galectin-3 and BIM was attenuated by Mst1 inactivation, but isoprenaline-induced galectin-3 expression was exaggerated by transgenic Mst1 activation. Pharmacological or genetic activation of β -adrenoceptors induced Mst1 expression and yes-associated protein (YAP) phosphorylation. YAP hyper-phosphorylation was also evident in Mst1 transgenic hearts with up-regulated expression of galectin-3 (40-fold) and BIM as well as up-regulation of many YAP-target genes by RNA sequencing. In H9c2 cells, isoprenaline

Abbreviations: BIM, Bcl-2 interacting mediator of cell death; dnMst1, dominant-negative Mst1; Gal-3, galectin-3; KO, knockout; Lats, large tumour suppression kinases; LV, left ventricle or left ventricular; Mst1, mammalian sterile-20 like kinase 1; nTG, non-transgenic; TG, transgenic; YAP, yes-associated protein

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induced YAP phosphorylation and expression of galectin-3 and BIM, effects simulated by forskolin but abolished by PKA inhibitors, and YAP knockdown induced expression of galectin-3 and BIM.

Conclusions and Implications: Stimulation of cardiac β -adrenoceptors activated the Mst1/Hippo pathway leading to YAP hyper-phosphorylation with enhanced expression of galectin-3 and BIM. This signalling pathway would have therapeutic potential.

LINKED ARTICLES: This article is part of a themed section on Adrenoceptors—New Roles for Old Players. To view the other articles in this section visit <http://onlinelibrary.wiley.com/doi/10.1111/bph.v176.14/issuetoc>

1 | INTRODUCTION

Activation of the sympatho- β -adrenergic system is a hallmark of heart disease and heart failure (Kaye et al., 1995; Triposkiadis et al., 2009). Stimulation of β -adrenoceptors leads to inotropic and lusitropic actions to maintain cardiac performance (Kaumann et al., 1999). However, in the setting of heart disease, sustained stimulation of β -adrenoceptors, due to enhanced sympathetic nervous activation with elevated catecholamine levels is associated with adverse prognosis (Cohn et al., 1984; Kaye et al., 1995). It is well known that sustained β -adrenoceptor stimulation leads to adverse cardiac effects notably fibrosis and cardiomyocyte apoptosis (Triposkiadis et al., 2009; Xiao et al., 2018). Understanding the mechanisms that drive myocardial fibrosis and apoptosis is essential for the development of new therapies.

Galectin-3 (Gal-3) is a β -galactoside-specific lectin that binds to intracellular and extracellular glycoproteins regulating their function particularly under diseased conditions (Meijers, Lopez-Andres, & de Boer, 2016; Nguyen et al., 2018; Takemoto et al., 2016). Clinically, Gal-3 is regarded as a biomarker predicting the risk of heart failure, atrial fibrillation, or all-cause mortality (Filipe, Meijers, Rogier van der Velde, & de Boer, 2015; Ghorbani et al., 2018). Meanwhile, Gal-3 is implicated as a causative mediator of cardiac inflammation and fibrosis (Nguyen et al., 2019; Rabinovich & Toscano, 2009; Suthahar et al., 2018; Takemoto et al., 2016; L. Yu et al., 2013). In the context of apoptosis, the Bcl-2 interacting mediator of cell death (BIM) is a BH3-only protein of the **Bcl-2** family and an essential initiator of apoptosis in diverse physiological and diseased settings (Bouillet & O'Reilly, 2009; Puthalakath et al., 2007). BIM senses pro-apoptotic signals and activates pro-apoptotic BAX and BAK while inhibiting anti-apoptotic proteins such as Bcl-2 and Mcl-2 (Bouillet & O'Reilly, 2009). We previously showed that cardiac expression of BIM is elevated by treatment with **isoprenaline** or myocardial ischaemia (Y. Y. Lee et al., 2013). Isoprenaline-induced apoptosis is abolished in hearts or cultured cardiomyocytes of BIM knockout (KO) mice (Y. Y. Lee et al., 2013). Collectively, there is good evidence for Gal-3 and BIM as potential therapeutic targets to inhibit cardiac fibrosis and apoptosis. However, the mechanism responsible for the up-regulated expression of both molecules in heart disease is unknown.

What is already known

- Stimulation of cardiac β -adrenoceptors induces expression of Gal-3 and BIM as pro-fibrotic and pro-apoptotic molecules.

What this study adds

- The β -adrenoceptor-Mst1(Hippo)-YAP signalling pathway mediated the up-regulated expression of Gal-3 and BIM in the heart.

What is the clinical significance

- Therapy with β -adrenoceptor antagonists in heart disease could inhibit this adverse β -adrenoceptor-Hippo signalling.
- The β -adrenoceptor-Mst1(Hippo) pathway provides a therapeutic target to down-regulate Gal-3 and BIM in heart disease.

We recently observed in transgenic (TG) mice with cardiac overexpression of mammalian sterile-20 like kinase 1 (Mst1) that expression of Gal-3 was elevated by approximately 50-fold (Nguyen et al., 2018). Being the mammalian ortholog of *Drosophila* Hippo kinase, Mst1 is the key kinase of the Hippo signalling pathway that is known to control organ size through regulation of cell proliferation and survival (F. X. Yu, Zhao, & Guan, 2015). Recent studies indicate a role of the Hippo pathway in diseased conditions such as tumour growth, heart disease, and regenerative medicine (Ikeda & Sadoshima, 2016; Leach et al., 2017; F. X. Yu et al., 2015). Studies have also reported coupling of GPCRs, including β -adrenoceptors, to the Hippo pathway (Kimura et al., 2016; F. X. Yu et al., 2012), although the downstream target genes that contribute to myocardial fibrosis and apoptosis remain unknown.

In the current study, our central hypothesis was that cardiac β -adrenoceptor stimulation up-regulated expression of Gal-3 and BIM through the Hippo signalling pathway. We have examined β -adrenoceptor-mediated activation of cardiac Mst1/Hippo pathway,

in relation to expression of Gal-3 and BIM and explored the role of YAP as the key transcription co-regulator. Our findings establish β -adrenoceptor-Mst1(Hippo)-YAP signalling as a controlling factor in the expression of Gal-3 and BIM in cardiac tissues.

2 | METHODS

2.1 | Animals

All animal care and experimental procedures in this study were approved by the local animal ethics committee in compliance with the Australian Code for the Care and Use of Animals for Scientific Purposes (8th edition) and animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010, McGrath & Lilley, 2015). Male C57BL/6J mice (12–16 weeks of age, RRID:IMSR_JAX:000664; from breeding stock at AMREP Animal Centre, Melbourne, Australia) were used. We used three TG strains of mice with transgenes driven by the α -myosin heavy chain promoter to achieve cardiomyocyte-specific expression of human β_2 -adrenoceptors (β_2 -TG; Milano et al., 1994), Mst1 (Mst1-TG), or dominant-negative mutant Mst1 (dnMst1-TG; Yamamoto et al., 2003). All strains of mice were on a C57BL/6J genetic background. Only male TG and non-transgenic (nTG) mice were studied. Age-matched non-TG littermates (nTG) were used as controls. Mice were housed in standard conditions with food and water provided ad libitum. Gal-3 gene deleted strain of mice (Gal-3 KO in C57BL/6J background) was obtained from Jackson Laboratories (Bar Harbor, ME). Mice were assigned randomly to different experimental groups.

In the β_2 -TG strain, cardiac β_2 -adrenoceptor density increases by approximately 200-fold (equivalent to 30-fold increase in density of total β -adrenoceptors; Milano et al., 1994; Xu et al., 2011). The β_2 -TG mice exhibit age-dependent development of cardiac fibrosis, cardiomyocyte loss, hypertrophy, ventricular arrhythmias, and heart failure (Du et al., 2000; Nguyen et al., 2015; Xu et al., 2011). With a 7.5-fold increase in Mst1 activity, Mst1-TG mice show early onset of dilated cardiomyopathy and severe interstitial fibrosis and are prone to atrial fibrillation (Nguyen et al., 2019; Pretorius et al., 2009; Yamamoto et al., 2003). Cardiomyocyte expression of kinase-dead mutant Mst1 (K59R) transgene acts as a dominant-negative inactivating endogenous Mst1 activity (Yamamoto et al., 2003). Both dnMst1-TG and Gal-3 KO strains of mice exhibit no cardiac phenotype under basal conditions. In all mouse strains used, body weight was comparable to respective wild-type littermates.

2.2 | Echocardiography and organ weights

Echocardiography was performed using an iE33 ultrasound system (Andover, MA) with images analysed in a blinded fashion (Donner, Kiriazis, Du, Marwick, & McMullen, 2018). Minipumps were removed to allow for washout of isoprenaline overnight, prior to imaging. Under isoflurane anaesthesia, a short-axis view of the left ventricle (LV) and 2D-guided M-mode traces were obtained. LV dimensions at diastole and systole were measured, and fractional shortening (FS) was

calculated. At the end, animals were killed by anaesthetic overdose (mixture of ketamine/xylazine/atropine at 200/40/2.4 mg·kg⁻¹, respectively, i.p.). Weights of the heart, LV, and lungs were obtained, and tibia length was determined.

2.3 | Hydroxyproline assay for collagen

Myocardial fibrosis was determined by hydroxyproline assay as previously described (Nguyen et al., 2015). An apical portion of the LV (about 20 mg) was used for the assay with hydroxyproline values converted into total collagen content. Results were normalized by dry weight of LV tissue.

2.4 | Cell experiments

Rat cardiomyoblasts H9c2 (RRID:CVCL_0286) were obtained from American Type Culture Collection (Rockville, MD, USA) and maintained in DMEM supplemented with 10% FBS and antibiotics. H9c2 cells grown to 70% confluency were treated with isoprenaline (3 μ M) for 48 hr or forskolin (10 μ M) for 24 hr. PKA inhibitors H89 (20 μ M) and PKI 14-22 (10 μ M) was added 30 min prior to isoprenaline treatment (3 μ M). After incubation, cells were harvested for analysis. Each experiment was done twice (testing isoprenaline, PKA inhibitors, or siRNA) or three times (testing forskolin).

2.5 | YAP silencing by small RNA interference

Small interfering RNA (siRNA)-mediated knockdown of YAP was performed in H9c2 cells according to manufacturer's instructions. Cells in 12-well plate were incubated with Acell siRNA delivery media (B-005000, Dharmacon, Lafayette, CO) containing a pool of target specific YAP-siRNA (Acell rat Yap1 363014 siRNA-SMARTpool, E-100439-00-0005, Dharmacon) or non-targeting control (Acell non-targeting siRNA #1, D-001919-01-05, 1 μ M, Dharmacon) at 37°C with 5% CO₂. After incubation for 72 hr, the cells were harvested for analysis.

2.6 | Protein expression

Total protein was extracted from LV tissues or cells. Tissues were homogenized with RIPA buffer (Cell Signaling Technology, Danvers, MA) with addition of 1-mM phenylmethanesulfonyl fluoride and proteinase or phosphatase inhibitor cocktails (#P8340 and #P5726, Sigma, St. Louis, MO). Cytoplasmic and nuclear fractions of protein were prepared as we previously described (Gao et al., 2011). Tissues were minced in homogenization buffer containing protease and phosphatase inhibitor cocktails and lysed by homogenization. Homogenates were centrifuged (5,000 \times g, 10 min at 4°C). The supernatants containing cytoplasmic proteins were collected. Pellets were carefully resuspended in a nuclear extraction buffer containing protease and phosphatase inhibitor cocktails. Quick freeze-thaw of pellet suspensions in liquid nitrogen was done three times, followed by centrifugation (12,000 \times g, 15 min at 4°C). The resulting supernatants containing nuclear protein were collected. Protein

concentration was determined using Pierce BCA protein assay kit (#23227, Thermo Scientific, Rockford, IL).

Protein level of Gal-3 in LV tissue was measured using the mouse Gal-3 DuoSet ELISA kit (DY1197; R&D Systems, Minneapolis, MN) according to manufacturer's instructions. The immunoblotting and other antibody-based procedures complied with the recommendations made by the *British Journal of Pharmacology*. Proteins (20–50 µg) were separated by 4–15% mini-PROTEAN TGX-Stain free gels (Bio-Rad, Hercules, CA) and blotted onto a PVDF membrane (Millipore, Billerica, MA). Using Bio-Rad TGX stain-free technology, the total protein signal was determined after blotting for normalization of target proteins. Membranes were blocked with 5% milk in TBST for 1 hr and incubated overnight (4°C) with the primary antibody against Mst1, p-YAP, YAP, Gal-3, or BIM (1:1,000), followed by incubation with the HRP-conjugated secondary antibodies. Membranes were developed with SuperSignal West Pico Plus Chemiluminescent substrate (#34580, Thermo Scientific, USA). Images were acquired with ChemiDoc Touch Imager (Bio-Rad) and quantified with Image Lab Software (version 4.5.2., RRID:SCR_014210, Bio-Rad).

2.7 | Gene expression by RT-PCR

RNA was extracted from LV tissue using Trizol[®] Reagent (Sigma). RNA (1 µg) was used for cDNA synthesis and quantitative PCR was performed using SYBR Green Master Mix qPCR (Roche, Mannheim, Germany) and target primers (Sigma). Gene expression level was normalized to that of GAPDH or TATA-Box Binding Protein Associated Factor 8 using the method of $2^{-\Delta\Delta C_t}$ and presented relative to that of control value.

2.8 | RNA sequencing and bioinformatics

LV tissues of 3-month-old Mst1-TG and nTG mice were homogenized in Trizol for RNA isolation using the Direct-zol[™] RNA MiniPrep Kit (Zymo Research, Irvine, CA); 200 ng RNA underwent ribosomal RNA depletion using the NEBNext[®] rRNA Depletion Kit followed by library construction using NEBNext[®] Ultra[™] Directional RNA Library Prep Kit for Illumina[®] (New England Biolabs, Ipswich, MA). Library QC was performed by Multi-NA bioanalyzer and then pooled to equimolar concentration. Pooled libraries underwent Illumina single read sequencing at the Australian Genome Research Facility (Melbourne, Australia) using HiSeq v4 reagents to generate 100 bp reads.

FastX Toolkit was used to trim low quality bases. Reads were mapped with STAR (Dobin et al., 2013) to the mouse genome downloaded from Ensembl (GRCm38, RRID:SCR_002344). FeatureCount (Liao, Smyth, & Shi, 2014) was used to count reads mapped to gene exons on the correct strand with a minimum mapping quality of 20 using Ensembl genome annotation (*Mus musculus*. GRCm38.85.gtf). Genes with fewer than 10 reads per sample on average were excluded. The resulting count matrix was analysed with the EdgeR package (Robinson, McCarthy, & Smyth, 2010). Genes were ranked from most up-regulated to most down-regulated by multiplying the sign of the \log_2 fold change by the inverse of the *P* value. Enrichment tests were performed using GSEA-P

(Subramanian, Kuehn, Gould, Tamayo, & Mesirov, 2007). YAP target genes were defined as having a peak within 1 kbp of a transcriptional start site according to YAP ChIP-seq data available from Cistrome DB (Lin et al., 2015).

2.9 | Data and statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018). Data are presented as mean ± SEM, unless otherwise stated. According to our previous studies and/or preliminary experiments on protein expression for intra-group variation and differences between group means, we calculated the group size and found that $n = 5$ was sufficient to detect a difference with 95% confidence and 80% power. All results were analysed using GraphPad Prism 7 Software (RRID:SCR_002798). Shapiro–Wilk's and Levene's tests were used to determine normality and equal variance, respectively. Data were tested by either one- or two-way ANOVA, followed by Bonferroni post hoc test. For datasets of only two groups, a Student's *t* test was used to compare differences. $P < .05$ was considered as statistically significant.

2.10 | Materials

Isoprenaline and **propranolol**, **carvedilol**, **atenolol**, and **ICI-118551** were supplied by Sigma. These compounds were dissolved in saline containing 0.4-mM ascorbic acid and administered through an osmotic minipump (ALZET model 2001, Cupertino, CA) subcutaneously implanted under isoflurane anaesthesia. Sigma also supplied forskolin (#F3917), the PKA inhibitor H89 (#B1427) and the PKA inhibitor, fragment 14–22 (PKI, #P9115). We used antibodies against Mst1 (Cell Signaling Technology Cat# 3682, RRID:AB_2144632), yes-associated protein (YAP, Cell Signaling Technology Cat#12395), p-YAP (ser127; Cell Signaling Technology Cat# 13008, RRID:AB_2650553), BIM (Cell Signaling Technology Cat# 2933, RRID:AB_1030947), Gal-3 (Thermo Fisher Scientific Cat# MA1-940, RRID:AB_2136775), Lamin A/C (E-1; Santa Cruz Biotechnology (Dallas, TX), Cat# sc-376248, RRID:AB_10991536), GAPDH (6C5; Santa Cruz Biotechnology Cat# sc-32233, RRID:AB_627679).

2.11 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander, Christopoulos et al., 2017; Alexander, Fabbro et al., 2017).

3 | RESULTS

3.1 | Pharmacological or genetic β -adrenoceptor activation up-regulates cardiac expression of Gal-3 and BIM

To investigate whether β -adrenoceptor activation affects cardiac expression of Gal-3 and BIM, isoprenaline was administered to C57BL/6J mice via osmotic minipump subcutaneously. Isoprenaline treatment increased expression of Gal-3 in a time- and dose-dependent fashion (Figure 1a). The isoprenaline-induced Gal-3 up-regulation

from the baseline level was inhibited by treatment with the non-selective β -antagonists, propranolol or carvedilol, by approximately 65% (Figure 1b). The selective β_1 -adrenoceptor antagonist atenolol or the β_2 -adrenoceptor antagonist ICI-118551, effectively inhibited isoprenaline-induced Gal-3 expression, and there was a trend towards a further reduction when combined (Figure 1c). Cardiac expression of BIM was increased after isoprenaline treatment in a time- and dose-dependent manner (Figure 1d), which was also inhibited by propranolol or carvedilol by approximately 70% (Figure 1e). Cardiac levels of Gal-3 and BIM were significantly higher in hearts from β_2 -TG mice than in those from nTG mice, at 4 and 8 months of age (Figure 1f,g).

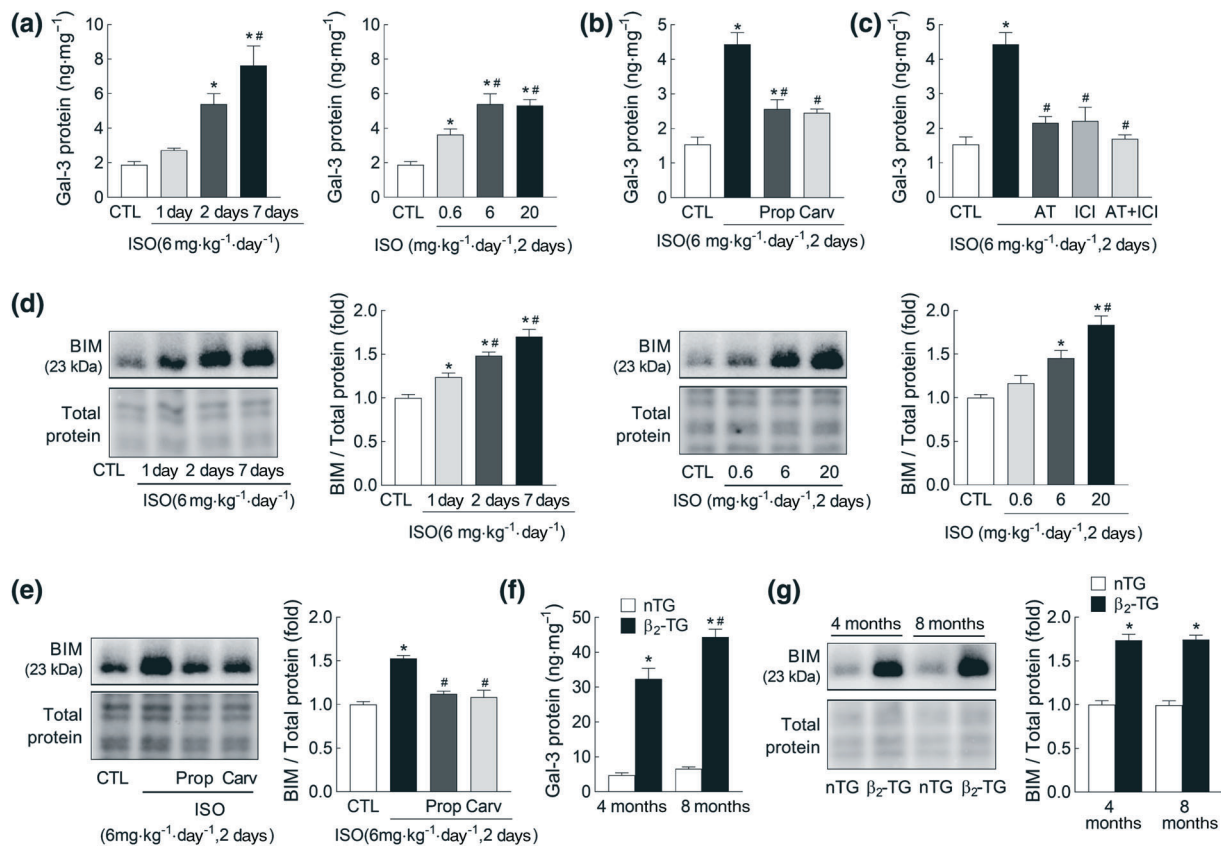


FIGURE 1 Pharmacological or genetic β -adrenoceptor activation up-regulates cardiac expression of Gal-3 and BIM. (a) Cardiac expression of Gal-3 in C57BL/6J mice treated with isoprenaline (ISO; $6 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$, s.c.) for 1, 2, or 7 days ($n = 5$ per group), respectively. $*P < .05$, significantly different from control (CTL), $\#P < .05$, significantly different from isoprenaline (1 day). Cardiac expression of Gal-3 in mice treated with isoprenaline for 2 days at 0.6, 6, or $20 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ ($n = 5$ per group), respectively. $*P < .05$, significantly different from CTL, $\#P < .05$, significantly different from isoprenaline ($0.6 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$, 2 days). (b, c) Cardiac expression of Gal-3 in mice treated for 2 days with isoprenaline alone ($6 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) or combined with non-selective β -adrenoceptor antagonist propranolol (Prop) or carvedilol (Carv, both at $2 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$), or with selective β_1 -adrenoceptor antagonist atenolol (AT, $2 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$), β_2 -adrenoceptor antagonist ICI-118551 (ICI, $1 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) or combination of both ($n = 6$ per group). $*P < .05$, significantly different from CTL, $\#P < .05$, significantly different from isoprenaline alone. (d) Cardiac expression of BIM in mice treated with isoprenaline ($6 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) for 1, 2, or 7 days ($n = 5$ per group), respectively. $*P < .05$, significantly different from CTL; $\#P < .05$, significantly different from isoprenaline (1 day). Cardiac expression of BIM in mice treated for 2 days with isoprenaline at 0.6, 6, or $20 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ ($n = 5$ per group), respectively. $*P < .05$, significantly different from CTL; $\#P < .05$, significantly different from isoprenaline ($0.6 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$). (e) Cardiac expression of BIM in mice treated for 2 days with isoprenaline alone ($6 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) or combined with propranolol (Prop) or carvedilol (Carv, both at $2 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$; $n = 5$ per group). $*P < .05$, significantly different from CTL, $\#P < .05$, significantly different from isoprenaline alone. (f, g) Cardiac expression of Gal-3 (f, $n = 9$ per group) or BIM (g, $n = 5$ per group) in nTG and β_2 -TG mice at 4 and 8 months of age. $*P < .05$, significantly different from age-matched nTG, $\#P < .05$, significantly different from 4-month β_2 -TG. Data shown are means \pm SEM. Results were compared by one-way (a-e) or two-way (f-g) ANOVA followed by Bonferroni post hoc test

3.2 | Pharmacological or genetic β -adrenoceptor activation induces Mst1 expression and YAP phosphorylation

We explored the mechanism by which β -adrenoceptor activation up-regulates Gal-3 and BIM focusing on Mst1 and YAP as key signalling components of the Hippo pathway. Relative to control value, cardiac protein level of Mst1 was increased by treatment with isoprenaline at 2 or 6 mg·kg⁻¹·day⁻¹ for 2 days or at 30 mg·kg⁻¹·day⁻¹ for 7 days (Figure 2a). In addition, isoprenaline treatment activated the Hippo signalling, as indicated by increased inhibitory Ser¹²⁷-phospho-YAP (p-YAP; Figure 2b,c), the downstream transcription co-regulator of the Hippo pathway. The isoprenaline-induced increase in p-YAP was strongly inhibited by treatment with propranolol or carvedilol (Figure 2d). Isoprenaline treatment increased total YAP by approximately 15–20%, while the increment of p-YAP was more pronounced (by 60%) leading to a 40% increase in the ratio of p-YAP/total YAP (Figure 2b–d).

Considering that the reduction in the nuclear YAP (nYAP) is a sign of YAP inactivation (Ikeda & Sadoshima, 2016), we determined total YAP as well as nYAP and cytoplasmic YAP (cYAP) by preparing respective protein fractions. GAPDH and Lamin A/C were used as marker proteins to ensure the good separation of the protein fractions (Figure 2e). In hearts of isoprenaline treated mice (6 mg·kg⁻¹·day⁻¹, 2 days), cYAP and nYAP normalized by total protein were all significantly increased with the increment of cYAP more pronounced (Figure 2e). nYAP as the ratio of cYAP was significantly lower in isoprenaline-treated group (Figure 2e).

Mst1 and YAP were also determined in β_2 -TG mouse hearts. Mst1 protein expression was significantly and age-dependently up-regulated in β_2 -TG hearts (Figure 3a). Increased p-YAP was observed in the β_2 -TG hearts (Figure 3b), indicating YAP inactivation in the setting of heightened β -adrenoceptor activity. In β_2 -TG hearts, total YAP was unchanged at 4 months of age but increased by 20% at 8 months of age, whereas p-YAP, normalized by either total protein or total YAP, was increased at both ages studied (Figure 3b).

3.3 | Mst1 is involved in β -adrenoceptor-mediated up-regulation of Gal-3 and BIM and YAP phosphorylation

The hearts from Mst1-TG mice exhibited increased expression of Gal-3 and BIM (Figure 4a,b), which was associated with enhanced YAP phosphorylation relative to nTG controls (Figure 4c). The role of Mst1 in mediating isoprenaline-induced up-regulation of Gal-3 was studied using mouse strains with cardiac Mst1 either inactivated (dnMst1-TG) or activated (Mst1-TG). Isoprenaline (6 mg·kg⁻¹·day⁻¹, 2 days) was given to dnMst1-TG, Mst1-TG mice, and their nTG littermates. Whereas isoprenaline treatment increased Gal-3 expression in nTG, dnMst1-TG, and Mst1-TG mice (Figure 4d), the absolute increment of Gal-3 by isoprenaline-stimulation, derived from individual values minus the group mean of respective controls, was

suppressed in dnMst1-TG heart by approximately 65% but exaggerated in Mst1-TG hearts by eightfold (Figure 4e), supporting the notion that isoprenaline-induced Gal-3 expression is Mst1-activity dependent.

The role of Mst1 in changes in expression of BIM and YAP hyper-phosphorylation induced by isoprenaline (6 mg·kg⁻¹·day⁻¹, 2 days) was determined in dnMst1-TG model. Isoprenaline-stimulated expression of BIM and YAP hyper-phosphorylation were abolished in the dnMst1-TG heart (Figure 4f,g).

3.4 | Role of PKA in isoprenaline-induced activation of Hippo signalling and expression of Gal-3 and BIM

We investigated, *in vitro*, the involvement of PKA in the β -adrenoceptor-mediated YAP phosphorylation and up-regulation of Gal-3 and BIM. Treatment of H9c2 cells with isoprenaline (3 μ M for 48 hr) induced YAP phosphorylation and up-regulation of both Gal-3 and BIM, which was abolished by the PKA inhibitors H89 (20 μ M; Figure 5a,b) or PKI (10 μ M; Figure 5c,d). Furthermore, treatment of H9c2 cells with the AC activator forskolin (10 μ M for 24 hr) significantly increased both YAP phosphorylation (Figure 5e) and expression of Gal-3 and BIM (Figure 5f).

With our finding of increased cardiac Mst1 expression in the heart of isoprenaline-treated mice, it remained unclear whether this effect of isoprenaline was PKA-dependent. This was tested in H9c2 cells treated with isoprenaline (3 μ M for 48 hr) or forskolin (10 μ M for 24 hr). Mst1 protein expression was significantly increased by treatment with isoprenaline (20% increase) or forskolin (60% increase; Figure 5g).

3.5 | YAP acts as transcription co-repressor for the expression of Gal-3 and BIM

Mst1-TG represents a model of YAP inactivation by the enhanced Mst1(Hippo) signalling. Changes in cardiac transcriptome profile was analysed by RNA sequencing of LV tissue from nTG ($n = 6$) and Mst1-TG ($n = 7$) mice. A total of 38.6 M reads per sample ($SD = 5.1$ M) were generated, and an average of 24.0 M reads was assigned to genes ($SD = 3.2$ M); 15,249 genes were expressed above the detection threshold of 10 reads per sample across all samples (RNA sequencing data uploaded to GEO:GSE106201). Relative to nTG, we identified in Mst1-TG heart a total of 8,619 differentially expressed genes ($FDR < 0.05$) with 4,080 genes up-regulated and 4,539 genes down-regulated (Figure 6a). Of the 666 YAP target genes expressed in this experiment according to publicly available ChIP-seq data, 262 were up-regulated and 179 were down-regulated (Figure 6b). This was further confirmed by GSEA enrichment analysis showing down- and up-regulation of YAP target genes (Figure 6c,d). This set of data suggests that YAP functions as transcription co-activator and co-repressor.

The role of YAP in regulating the expression of Gal-3 and BIM was studied *in vitro*. H9c2 cells were transfected with YAP siRNA (or non-targeting siRNA as control) for 72 hr. siRNA transfection

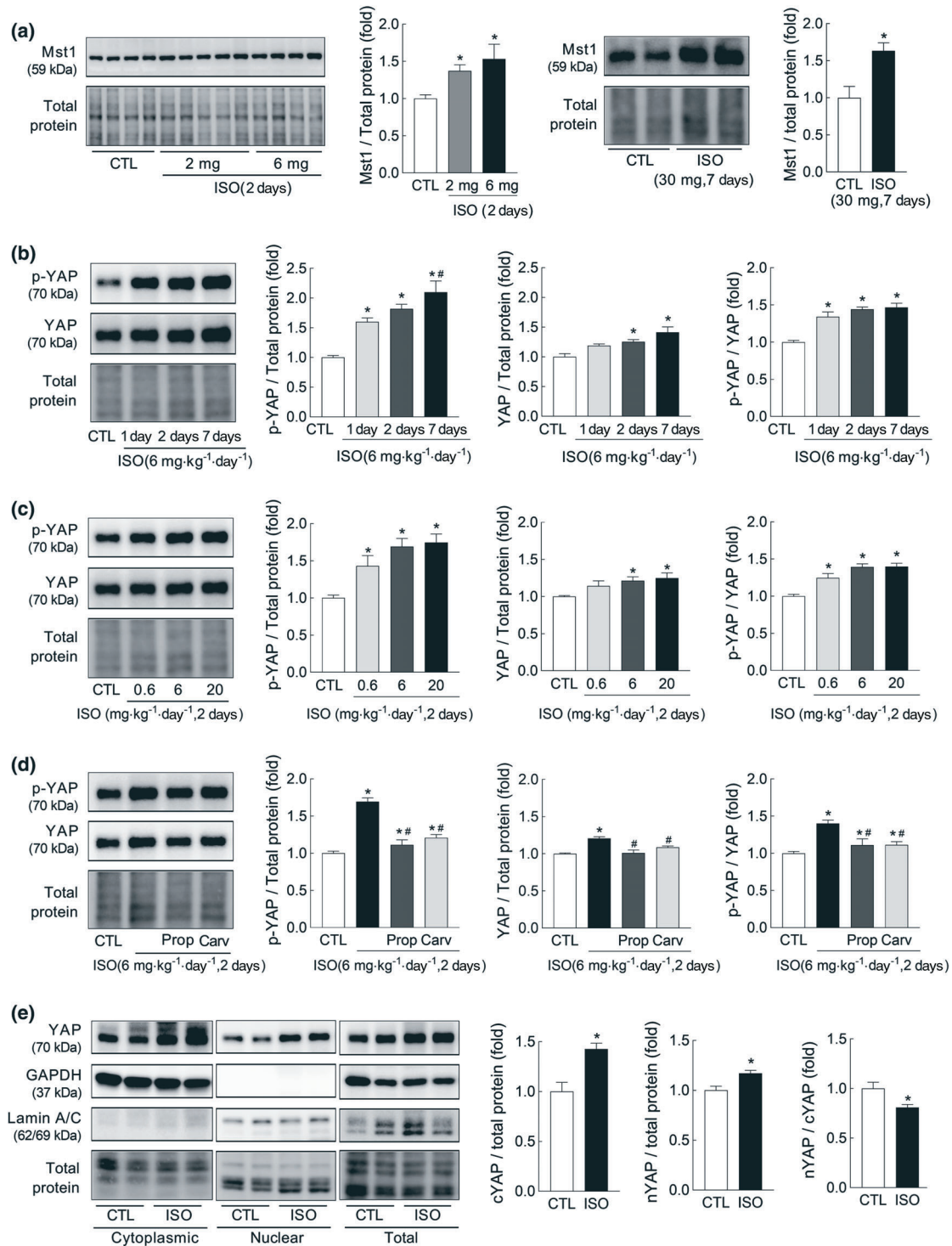


FIGURE 2 Pharmacological β -adrenoceptor stimulation induces cardiac Mst1 expression and YAP phosphorylation. (a) Cardiac protein expression of Mst1 in C57BL/6J mice treated with isoprenaline (ISO, 2 or 6 mg·kg⁻¹·day⁻¹, 2 days; 30 mg·kg⁻¹·day⁻¹, 7 days). * $P < .05$, significantly different from CTL. (b) Cardiac protein expression of phosphorylated YAP (p-YAP) and total YAP, and p-YAP/YAP ratio in mice treated with isoprenaline (6 mg·kg⁻¹·day⁻¹) for 1, 2, or 7 days, respectively. * $P < .05$, significantly different from CTL; # $P < .05$, significantly different from isoprenaline (1 day). (c) Expression of p-YAP, total YAP, and p-YAP/YAP ratio in hearts of mice treated with isoprenaline for 2 days at 0.6, 6, or 20 mg·kg⁻¹·day⁻¹, respectively. * $P < .05$, significantly different from CTL. (d) Cardiac expression of p-YAP and total YAP and p-YAP/YAP ratio in mice treated for 2 days with isoprenaline (6 mg·kg⁻¹·day⁻¹, 2 days) alone or combined with propranolol (Prop, 2 mg·kg⁻¹·day⁻¹) or carvedilol (Carv, 2 mg·kg⁻¹·day⁻¹). * $P < .05$, significantly different from CTL; # $P < .05$, significantly different from isoprenaline. (e) Expression of YAP of cytoplasmic (cYAP), nuclear (nYAP) and total protein fractions, and nYAP/cYAP ratio in hearts of isoprenaline-treated mice (6 mg·kg⁻¹·day⁻¹, 2 days). * $P < .05$, significantly different from CTL. All data sets, $n = 5$ per group. Data shown are means \pm SEM and were compared by two-tailed Student's t test (a, e) or one-way ANOVA (a-d), followed by Bonferroni post hoc test

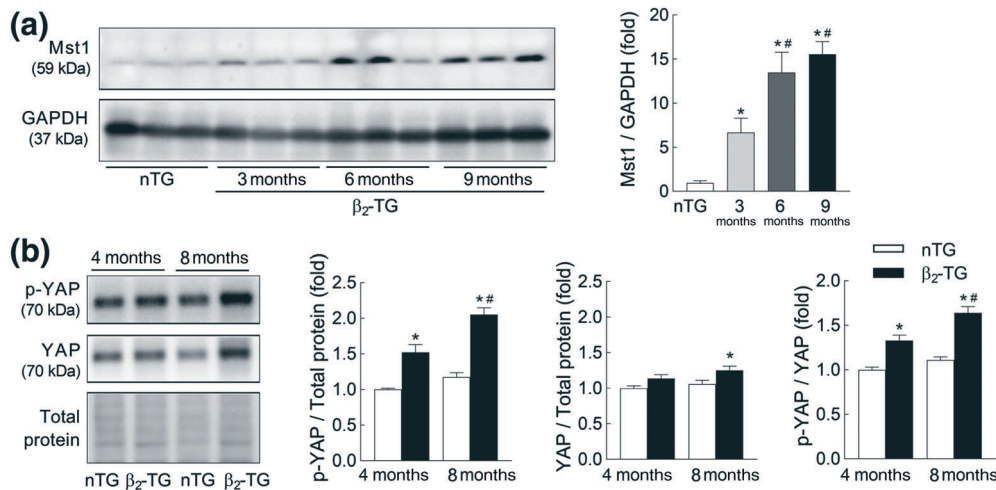


FIGURE 3 Genetic activation of β_2 -adrenoceptors induced Mst1 expression and YAP phosphorylation. (a) Up-regulation of Mst1 protein in the β_2 -TG hearts at 3, 6, and 9 months of age. $n = 6$ per group. * $P < .05$, significantly different from nTG, # $P < .05$ versus 3-month β_2 -TG. (b) expression of cardiac p-YAP, total YAP, and p-YAP/YAP ratio in nTG and β_2 -TG mice at 4 and 8 months of age ($n = 5$ per group). * $P < .05$, significantly different from age-matched nTG, # $P < .05$, significantly different from 4-month β_2 -TG. Data shown are means \pm SEM and were compared by one-way (a) or two-way (b) ANOVA, followed by Bonferroni post hoc test

reduced YAP protein abundance by about 80% together with a significant increase in the expression of Gal-3 and BIM (Figure 6e), implying that YAP acts as transcription co-repressor for the expression of both genes.

3.6 | Influence of Gal-3 on expression of YAP-target genes

We determined the expression of three YAP-target genes, that is, connective tissue growth factor (CTGF), ankyrin repeat domain 1 (Ankrd1), and baculoviral IAP repeat containing 5 (Birc5), with their expression known to be activated by YAP (Chen et al., 2014; Matsuda et al., 2016; Plouffe et al., 2016). These selected genes were consistently up-regulated in the heart of isoprenaline-treated mice or Mst1-TG mice (Figure 7a). The effects of Gal-3 on the expression of these genes was studied. Specifically, expression of these genes was determined in LV tissues from wild-type control or Gal-3 KO mice treated with isoprenaline ($30 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$, 7 days) or from mice generated by crossing Mst1-TG and Gal-3 KO strains (i.e., nTG, Gal-3 KO, Mst1-TG, and Mst1-TG/Gal-3 KO; Nguyen et al., 2019). In both settings, up-regulated expression of these genes, except Birc5 in the Mst1-TG background, was significantly blunted by Gal-3 gene deletion (Figure 7b,c).

3.7 | ISO induced cardiotoxicity and effect of Gal-3 gene deletion

Our previous study showed that isoprenaline-induced cardiomyocyte apoptosis is dependent on the induction of BIM (Y. Y. Lee et al., 2013). Here, we studied the effect of Gal-3 in isoprenaline-induced cardiotoxicity. In wild-type control mice, isoprenaline treatment ($30 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) for 7 days induced a 40% reduction in LV FS

(Figure 8a), a 20% increase in wet weight of the LV or whole heart indicating cardiac hypertrophy without change in the lung weight (Figure 8a). isoprenaline-treated wild-type mice showed eightfold increase in Gal-3 expression at mRNA and protein levels (Figure 8b) and increased expression of inflammatory or fibrotic genes (Figure 8c,d), changes associated with a 55% increase in the LV collagen content (Figure 8d). Gal-3 gene deletion was cardioprotection against isoprenaline cardiotoxicity on the majority of the parameters except for hypertrophy (i.e., LV and heart weight; Figure 8a–d).

4 | DISCUSSION

A few major findings are made in this study. First, cardiac β -adrenoceptor activation by pharmacological or genetic means increases expression of both pro-fibrotic Gal-3 and pro-apoptotic BIM, changes associated with increased Mst1 expression and YAP phosphorylation. These effects of isoprenaline were inhibited by β -adrenoceptor antagonists. Second, genetic Mst1 inactivation inhibited the isoprenaline-induced Ser¹²⁷-hyper-phosphorylation of YAP and up-regulation of both Gal-3 and BIM. Conversely, transgenic Mst1 activation per se induces hyper-phosphorylation of YAP and up-regulation of Gal-3 and BIM and augmented isoprenaline-induced Gal-3 expression. Third, our in vitro and in vivo data indicate that β -adrenoceptor activation leads to PKA-dependent increase in Mst1 expression, YAP phosphorylation, and up-regulated expression of Gal-3 and BIM. Finally, up-regulation of Gal-3 as a consequence of activated β -adrenoceptor-Mst1(Hippo) signalling mediates isoprenaline-cardiotoxicity.

In mouse hearts in vivo, pharmacological stimulation of β -adrenoceptor induces up-regulation of Gal-3 protein in a time-dependent fashion occurring within 48 hr, as well as in a dose-dependent manner including a pharmacological dosage ($0.6 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$). Under

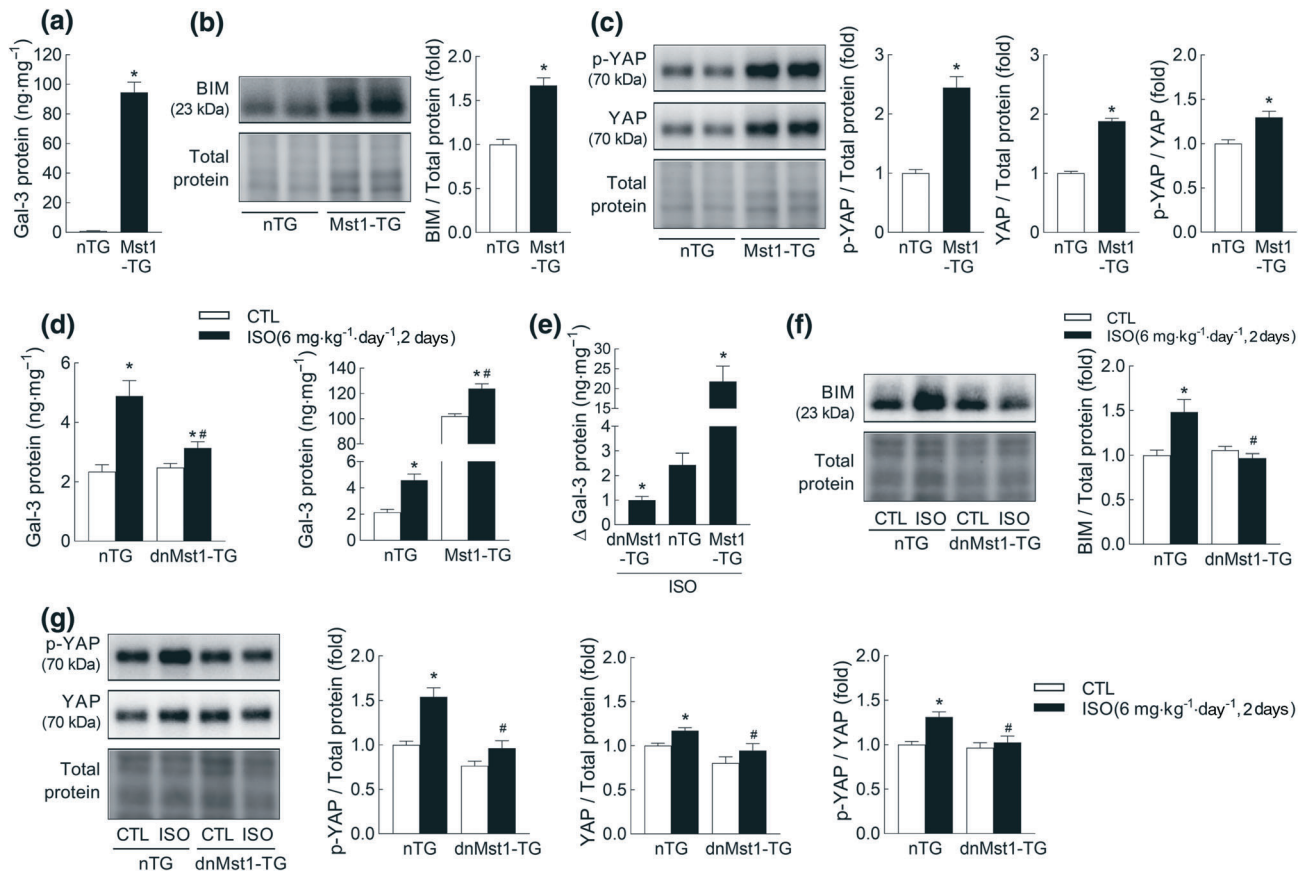


FIGURE 4 Mst1 is involved in β -adrenoceptor-mediated YAP phosphorylation and up-regulation of Gal-3 and BIM. Expression of Gal-3 (a, $n = 8$ per group) and BIM (b, $n = 6$ per group) in nTG and Mst1-TG hearts. * $P < .05$, significantly different from nTG. (c) Protein expression of p-YAP, total YAP, and p-YAP/YAP ratio in nTG and Mst1-TG hearts. * $P < .05$, significantly different from nTG ($n = 6$ per group). (d) Cardiac expression of Gal-3 in dnMst1-TG and Mst1-TG and respective nTG mice treated with isoprenaline (ISO, 6 mg·kg⁻¹·day⁻¹, 2 days). * $P < .05$, significantly different from respective CTL; # $P < .05$, significantly different from nTG + isoprenaline. (e) Absolute changes of Gal-3 content by isoprenaline treatment in nTG, dnMst1-TG, and Mst1-TG mice. * $P < .05$, significantly different from nTG + isoprenaline (6 mg·kg⁻¹·day⁻¹, 2 days). All data sets in d and e, $n = 8$ per group. (f) Cardiac expression of BIM in nTG and dnMst1-TG mice treated with isoprenaline (6 mg·kg⁻¹·day⁻¹, 2 days; $n = 5$ per group). * $P < .05$, significantly different from respective CTL; # $P < .05$, significantly different from nTG + isoprenaline. (g) Protein levels of p-YAP and YAP in nTG and dnMst1-TG mice treated with isoprenaline (6 mg·kg⁻¹·day⁻¹, 2 days; $n = 5$ per group). * $P < .05$, significantly different from respective CTL; # $P < .05$, significantly different from nTG + isoprenaline. Data shown are means \pm SEM and were compared by two-tailed Student's *t* test (a–c), one-way (e) or two-way (d, f, g) ANOVA, followed by Bonferroni post hoc test

our experimental conditions, isoprenaline stimulation also increased protein expression of BIM, a finding consistent with our previous report (Y. Y. Lee et al., 2013). We showed that isoprenaline-induced cardiac expression of Gal-3 and BIM was significantly inhibited by the concomitant treatment with non-selective β -adrenoceptor antagonists. These findings imply that Gal-3 and BIM as potential therapeutic targets to suppress β -adrenoceptor-mediated cardiac fibrosis and apoptosis. Furthermore, we found that isoprenaline-induced Gal-3 expression was suppressed not only by non-selective β -adrenoceptor antagonists but also by selective β -adrenoceptor antagonists, atenolol and ICI-118551 with only a trend for further inhibition when combined. This might be explained by similar Gsa/cAMP/PKA coupling of β_1 - and β_2 -adrenoceptor mediating similar cellular excitation (Kaumann et al., 1999; Kaumann & Lemoine, 1987). This is particularly true in the diseased myocardium where β_2 -adrenoceptors are redistributed, thereby mediating a broader signalling than the β_1 -adrenoceptors (Nikolaev

et al., 2010; Wright et al., 2014). In addition, ICI-118551 at 0.1 μ M or higher concentrations will block effects mediated by β_1 -adrenoceptors (Kaumann & Lemoine, 1987; O'Donnell & Wanstall, 1980) or chronotropic response to sympathetic nerve stimulation in perfused mouse hearts (Du et al., 1996).

BIM is an essential initiator of apoptosis (Bouillet & O'Reilly, 2009; Puthalakath et al., 2007). We previously showed that BIM gene deletion is cardioprotective against isoprenaline-induced cardiotoxicity (Y. Y. Lee et al., 2013). Specifically, with chronic isoprenaline treatment, BIM KO mice were resistant, relative to wild-type controls, to isoprenaline-induced cardiomyocyte apoptosis, hypertrophy, and ventricular dysfunction. In Gal-3 KO mice, cardiac damage induced by treatment with isoprenaline for 7 days was abolished as shown by the absence of LV dysfunction and fibrosis and blunted fibrotic and inflammatory signalling. Thus, Gal-3 and BIM are critical molecules in mediating cardiotoxicity induced by β -adrenoceptor stimulation.

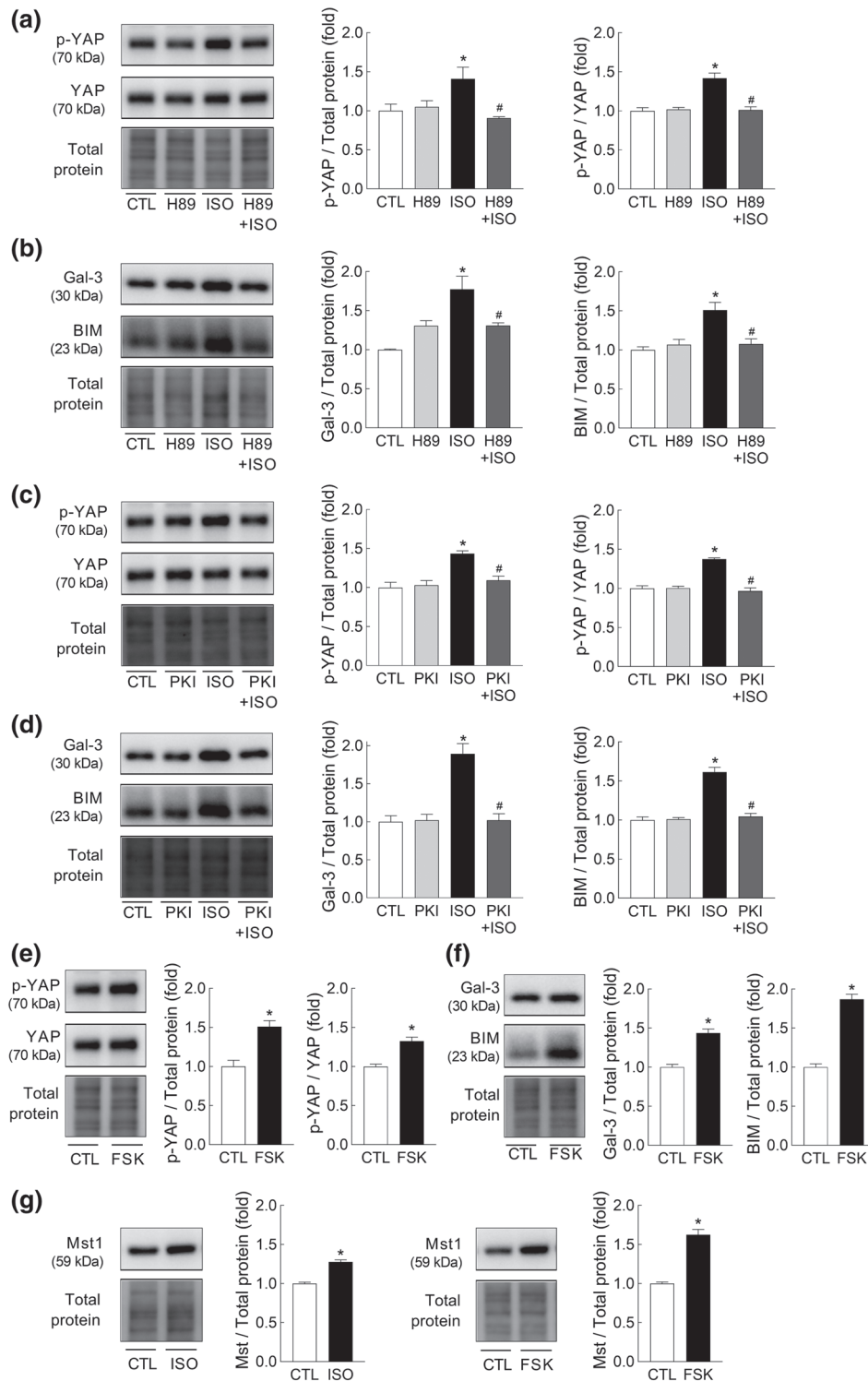


FIGURE 5 PKA is involved in β -adrenoceptor-induced YAP phosphorylation as well as Gal-3 and BIM up-regulation in H9c2 cells. H9c2 cells were pretreated with H89 (20 μ M) or PKI 14–22 (10 μ M) for 30 min prior to addition of isoprenaline (ISO; 3 μ M) for 48 hr. Panels a and b are representative immunoblotting images and grouped data of p-YAP and YAP (a) or Gal-3 and BIM (b) in H9c2 cells after isoprenaline treatment alone or combined with H89. Panels c and d are representative immunoblotting images and grouped data of p-YAP and p-YAP/YAP ratio (c) or Gal-3 and BIM (d) in H9c2 cells after isoprenaline treatment alone or combined with PKI. For panels a–d, * P < .05, significantly different from CTL, # P < .05, significantly different from isoprenaline alone. H9c2 cells were treated with the AC activator forskolin (FSK, 10 μ M) for 24 hr. Panels e and f show representative immunoblotting images and grouped data of p-YAP and YAP (e) or protein expression levels of Gal-3 and BIM (f). (g) Effect of isoprenaline (3 μ M, 48 hr) or forskolin (10 μ M, 24 hr) on Mst1 protein expression. For panels e–g, * P < .05, significantly different from CTL. For all data sets, n = 5 per group. Data shown are means \pm SEM and were compared by two-tailed Student's t test (e–g) or one-way ANOVA (a–d) followed by Bonferroni post hoc test

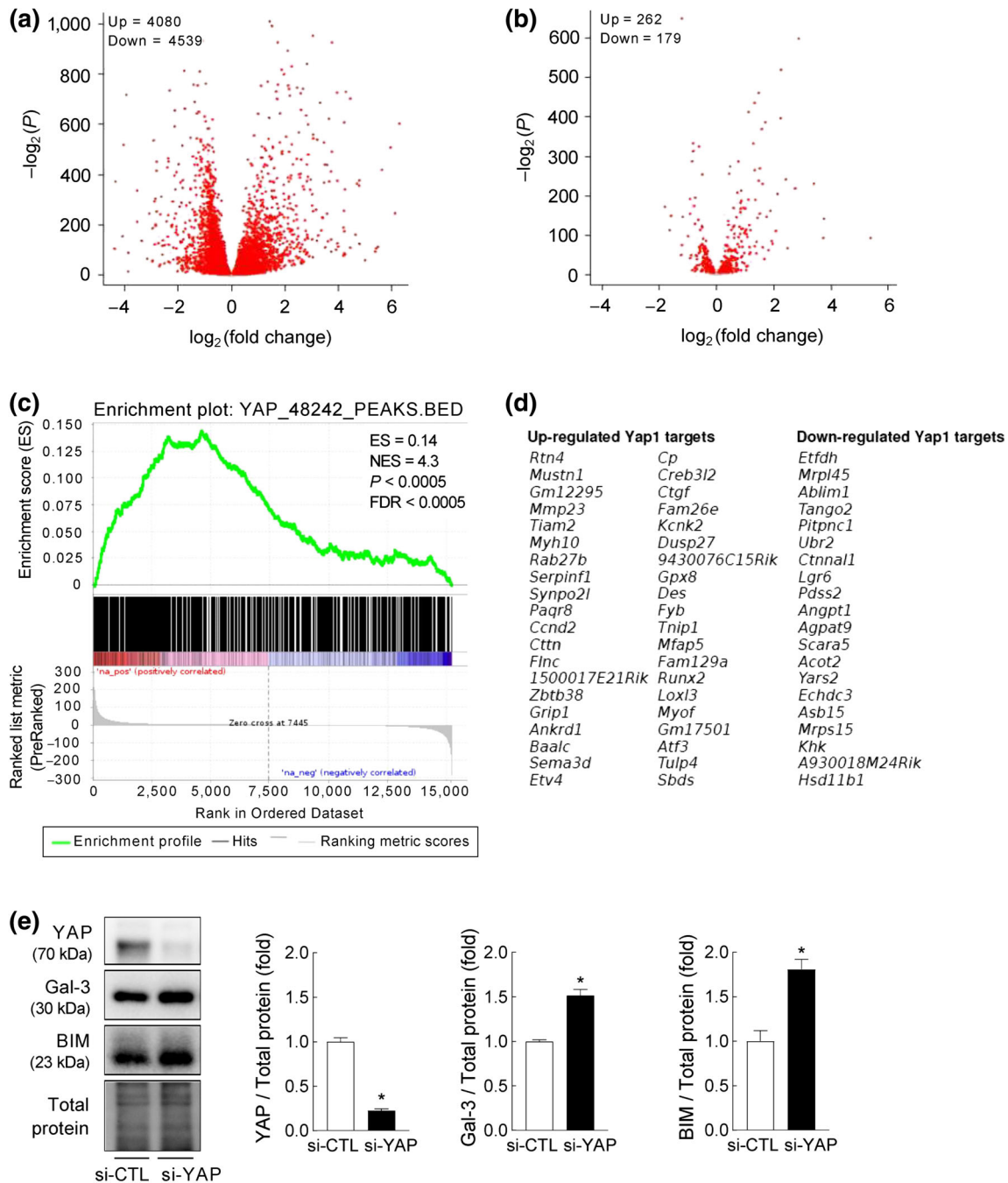


FIGURE 6 YAP is transcription co-activator and co-repressor, and its inhibition is associated with up-regulation of Gal-3 and BIM. Panels a–d show changes in cardiac gene expression by Mst1 overexpression. (a) Volcano plot of total RNA sequencing showing differentially regulated genes (DEGs) of Mst1-TG versus nTG hearts. (b) Volcano plot of YAP target genes according to ChIP-seq data. Activation of the Mst1(Hippo) signalling in the Mst1-TG heart leads to down- and up-regulated YAP target genes. For panels a and b, red points denote statistical significance (FDR < 0.05). (c) GSEA enrichment plot shows quantitatively that YAP target genes are collectively up-regulated in the Mst1-TG heart ($P < .0005$). (d) List of top down- and up-regulated YAP-target genes in Mst1-TG hearts relative to nTG values. For panels a–d, $n = 6$ for nTG group and $n = 7$ for TG group. (e) H9c2 cells were transfected with YAP-siRNA or control-siRNA (1 μ M for 72 hr). Representative immunoblotting images and changes in protein expression levels of YAP, BIM, and Gal-3. * $P < .05$, significantly different from CTL ($n = 6$ per group). Data shown are means \pm SEM and were compared by two-tailed Student's t test

Similar to isoprenaline treatment, up-regulated expression of Gal-3 and BIM was found in the β_2 -TG mouse heart. Furthermore, we also showed elevated cardiac expression of Mst1 by isoprenaline treatment or in β_2 -TG hearts. These findings guided us to explore

the possibility that expression of Gal-3 and BIM is regulated by the Mst1(Hippo) signalling pathway. The effector of the Hippo pathway is the transcription co-regulator YAP and its paralog TAZ (transcriptional coactivator with PDZ-binding motif) controlling

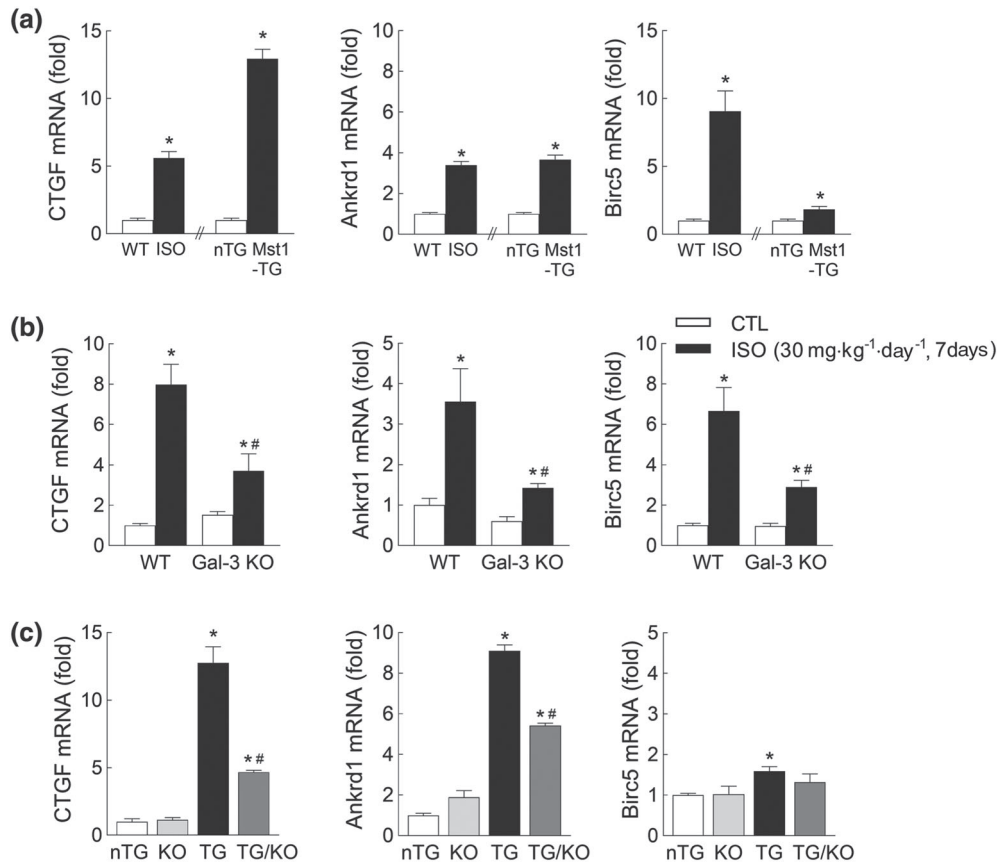


FIGURE 7 Changes in cardiac expression of selected YAP-target genes by isoprenaline treatment or Mst1 overexpression and effect of Gal-3 gene deletion. (a) Cardiac expression of YAP-target genes in wild-type mice treated with isoprenaline (ISO; 6 mg·kg⁻¹·day⁻¹, 2 days) or in Mst1-TG mice (n = 6 per group). *P < .05, significantly different from respective controls. (b) Cardiac expression of YAP-target genes in mice with or without isoprenaline treatment (ISO; 30 mg·kg⁻¹·day⁻¹, 7 days) and effect of Gal-3 gene deletion (Gal-3 KO; n = 5 per group). *P < .05, significantly different from control of the same genotype, #P < .05, significantly different from WT + isoprenaline. (c) Expression of YAP target genes in hearts of Mst1-TG (TG) and littermate control mice (i.e., nTG and Gal-3 KO) and effect of Gal-3 gene deletion (i.e., TG/KO; n = 5 per group). *P < .05, significantly different from age-matched nTG, #P < .05, significantly different from TG. Data shown are means ± SEM and were compared by two-tailed Student's *t* test (a), two-way (b) or one-way (c) ANOVA, followed by Bonferroni post hoc test

expression of a number of target genes responsible for cell proliferation and survival (F. X. Yu et al., 2015). Activation of the Hippo signaling pathway leads to YAP/TAZ phosphorylation and inactivation (F. X. Yu et al., 2015). In the present study, we consistently observed YAP Ser¹²⁷-hyper-phosphorylation in models with enhanced β-adrenoceptor activity (i.e., isoprenaline treatment and β₂-TG) or with Mst1 activation (i.e., Mst1-TG). Although YAP is well known as a transcription co-activator, we found, in H9c2 cells, that siRNA-mediated YAP knockdown led to significant up-regulation of Gal-3 and BIM. In the Mst1-TG hearts, numerous genes were differentially expressed relative to nTGs with roughly equal number of up- and down-regulated genes, including a number of YAP-target genes. In fact, 59% of the YAP-target genes were up-regulated in the Mst1-TG heart with YAP inactivation. Thus, we propose that for cardiac expression of Gal-3 and BIM, YAP acts as a transcription co-repressor and represents a STOP signal. Recent studies in different cell lines have indicated YAP as transcription co-repressor (Kim, Kim, Johnson, & Lim, 2015; Valencia-Sama et al., 2015).

Yu et al. firstly reported that the Hippo pathway as a downstream branch of GPCR signalling (F. X. Yu et al., 2012). They demonstrated in cell lines that activation of G_s-coupled receptors, including glucagon receptors or β₂-adrenoceptors, induced YAP phosphorylation, which is dependent on Rho kinase but independent of Mst1 (F. X. Yu et al., 2012). Our findings in H9c2 cells indicate that activation of the β-adrenoceptor-Mst1(Hippo) signalling involves the AC-cAMP-PKA signalling pathway. In the current study, we observed increment of Mst1 expression by both isoprenaline and forskolin, indicating involvement of PKA. Treatment with forskolin also stimulated YAP hyper-phosphorylation and expression of Gal-3 and BIM, effects similar to that of isoprenaline in vitro and in vivo. Conversely, inhibition of PKA using H89 or PKI alleviated β-adrenoceptor-stimulated YAP hyper-phosphorylation as well as up-regulation of Gal-3 and BIM. Collectively, these data suggest involvement of the AC/cAMP/PKA pathway in β-adrenoceptor-Mst1(Hippo) signalling that regulates expression of Gal-3 and BIM. It is well recognized that chronic β-adrenoceptor stimulation occurs in models of heart disease due to

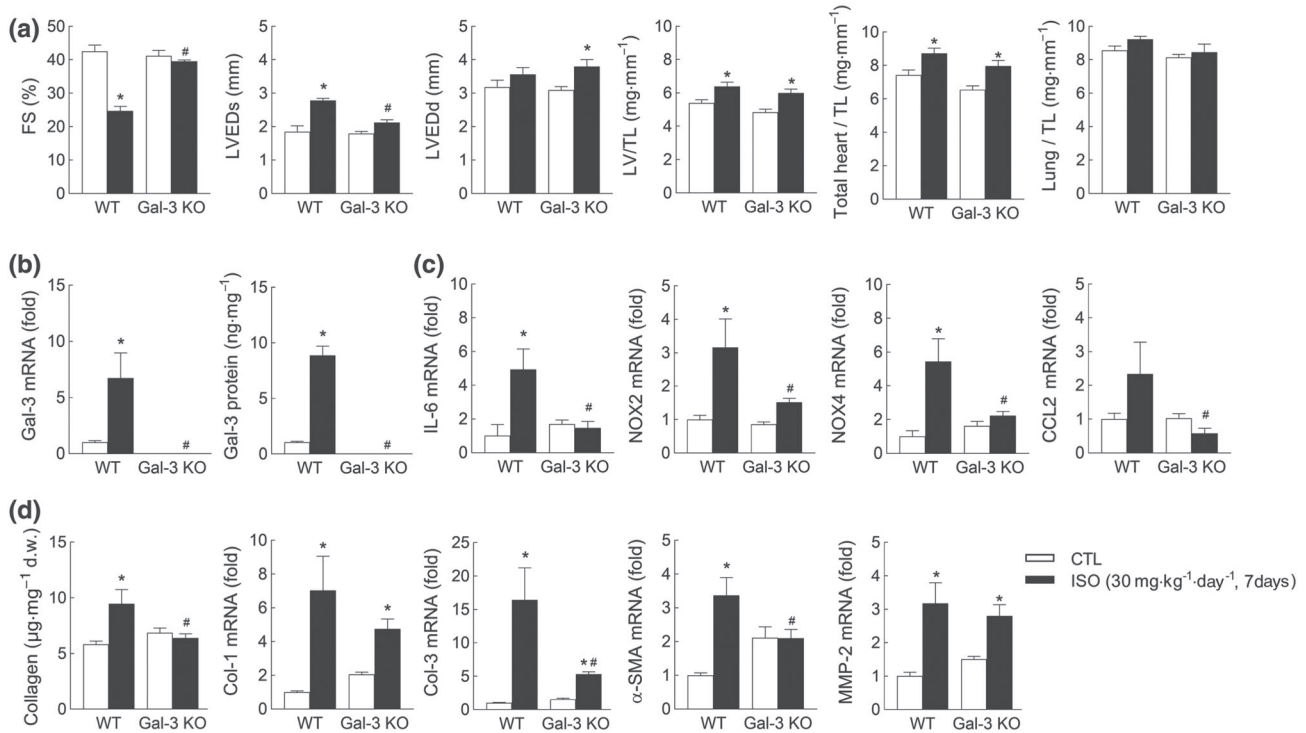


FIGURE 8 Isoprenaline-induced cardiotoxicity and effect of Gal-3 gene deletion. Wild-type (WT) and Gal-3 knockout (KO) mice were treated with isoprenaline (ISO; 30 mg·kg⁻¹·day⁻¹, 7 days). (a) Echocardiography-derived left ventricular (LV) fractional shortening (FS), LV dimensions at end-systole (LVEDs), or end-diastole (LVEDd) and wet weights of the LV, whole heart, and lungs normalized by tibia length (TL). (b) Expression of Gal-3 at mRNA and protein levels. (c) Expression of selected inflammatory genes. (d) LV content of collagen by hydroxyproline assay and expression of selected fibrotic genes. For all data sets, $n = 6$ per group. * $P < .05$, significantly different from control of the same genotype, # $P < .05$, significantly different from WT + isoprenaline. Data shown are means \pm SEM and were compared by two-way ANOVA, followed by Bonferroni post hoc test. Col, procollagen; α SMA, α -smooth muscle actin; NOX, NADPH oxidase

almost any aetiology leading to cardiac apoptosis and fibrosis (G. J. Lee, Yan, Vatner, & Vatner, 2015; Peter et al., 2007; Xiao et al., 2018; Xu et al., 2011). Our study provides the mechanistic link by showing the coupling of β -adrenoceptors with the Hippo pathway as well as up-regulated Gal-3 and BIM as the target genes. Currently, the potential transcription factors that orchestrate with YAP in mediating up-regulation of Gal-3 and BIM remain undefined. Numerous transcription factors have been implicated in interacting with YAP to regulate target genes (Ikeda & Sadoshima, 2016; J. Wang, Liu, Heallen, & Martin, 2018), and we previously showed in the heart that isoprenaline-induced up-regulation of BIM requires binding of cMyc and CREB-binding protein to BIM promoter region (Y. Y. Lee et al., 2013).

In the present study, we addressed the Mst1-dependency of cardiac β -adrenoceptor-mediated activation of the Hippo pathway by investigating dnMst1-TG model (Yamamoto et al., 2003). Relative to the nTG littermates, isoprenaline-induced YAP hyper-phosphorylation together with up-regulation of Gal-3 and BIM were significantly suppressed in dnMst1-TG hearts, while isoprenaline-induced Gal-3 expression was exaggerated in Mst1-TG hearts. Consistent with this finding is the study by Lee et al. showing that Mst1 inhibition in β_1 -adrenoceptor TG mice by crossing with dnMst1-TG mice rescued cardiomyopathy with suppressed myocardial inflammatory, fibrosis,

premature death, and LV dysfunction (G. J. Lee et al., 2015). Cardiomyocytes from Mst1-KO mice also show increased resistance to isoprenaline-induced cardiomyocyte apoptosis (G. J. Lee et al., 2015). The present study and previous studies by Vatner's team (G. J. Lee et al., 2015; Peter et al., 2007) have shown that chronic treatment with isoprenaline or transgenic activation of cardiac β -adrenoceptor (i.e., β_1 -TG or β_2 -TG) leads to increased expression of Mst1. Together with the YAP hyper-phosphorylation in Mst1-TG hearts, we have provided in vivo evidence that cardiac β -adrenoceptor activation induces YAP phosphorylation via the classic Hippo pathway involving Mst1.

Our finding of YAP hyper-phosphorylation in the Mst1-TG heart implies that increased abundance and activity of Mst1 drives a sustained activation of the Hippo signalling pathway with inactivation of transcriptional activity of YAP. Cardiac-restricted overexpression of Mst1 leads to cardiomyocyte apoptosis and myocardial fibrosis (Yamamoto et al., 2003). Our recent data from RNA sequencing of Mst1-TG hearts revealed up-regulation of the Hippo pathway-related genes, including Lats, YAP, and Mob1 (Nguyen et al., 2019). The extent of Gal-3 up-regulation is the highest in the Mst1-TG heart, relative to all reported preclinical heart disease models (Nguyen et al., 2019; Takemoto et al., 2016; L. Yu et al., 2013). We recently showed that Gal-3 gene deletion in the Mst1-TG mice is anti-fibrotic

together with alleviation of cardiac dilatation and dysfunction, adding mechanistic insight into fibrogenesis in this model of dilated cardiomyopathy (Nguyen et al., 2019).

Increased p-YAP in our models indicates YAP inactivation. Unexpectedly, abundance of nYAP, another marker of YAP activity, was increased albeit nYAP/cYAP ratio is lower in isoprenaline treated hearts. Importantly, the three models studied all had increased abundance of total YAP, which is distinct from many other studies showing that activated Hippo signalling increases ratio of p-YAP/total YAP or nYAP/cYAP without change in total YAP (Del Re et al., 2013; Kimura et al., 2016; Plouffe et al., 2016; L. Wang et al., 2016; F. X. Yu et al., 2012). In addition to YAP, Mst1 expression is also up-regulated by isoprenaline treatment or in β_2 -TG hearts, and we recently observed up-regulated gene expression of the components of Hippo signalling by RNA sequencing in Mst1-TG model (Nguyen et al., 2019). However, Mst1-TG heart also showed concomitant gene up-regulation of factors opposing the Hippo signalling (Nguyen et al., 2019). Of them, Amot and Amotl1 act as YAP/TAZ inhibitors (Chan et al., 2011) and YWHAB and YWHAE code for 14-3-3 involved in YAP cytoplasmic retention (Habbig et al., 2011). Thus, in the diseased heart *in vivo*, we reveal a unique and complex situation where sustained β -adrenoceptor stimulation increases protein abundance of many Hippo signalling molecules. Further study is required to explore the mechanism(s) responsible for the up-regulation of Hippo components including YAP and the ultimate effect on YAP transcriptional activity in the setting of sustained activation of β -adrenoceptor-(Mst1)Hippo signalling.

Our study is the first to demonstrate that Gal-3 and BIM, two genes known to contribute to heart disease, are under regulation by the Hippo-YAP signalling, finding that would improve our understanding on the role of the Hippo pathway in heart disease. Based on our findings of up-regulation of Gal-3 and BIM due to enhanced cardiac β -adrenoceptor-Mst1(Hippo) signalling, a maintained YAP activity would be cardioprotective. This view is indeed supported by relatively recent clinical and preclinical studies. In myocardial biopsy of human patients with cardiomyopathy, YAP hyper-phosphorylation is evident indicating activation of the Hippo signalling pathway (Chen et al., 2014; Leach et al., 2017). Conditional deletion of YAP in embryonic mouse hearts results in myocardial hypoplasia and embryonic lethality (Xin et al., 2013), while cardiomyocyte-specific deletion of YAP leads to cardiomyocyte apoptosis, dilated cardiomyopathy, and lethality (Del Re et al., 2013; Xin et al., 2013). Under conditions of chronic myocardial infarction, inactivation of the Hippo pathway by means of deleting Salvador (Leach et al., 2017) or Lats (Heallen et al., 2013), expressing either mutant dnMst1 gene (Odashima et al., 2007) or mutant dominant-active YAP gene (Lin et al., 2014), has been shown to lead to better preservation of cardiac function due to improved myocardial regeneration and reduced fibrosis. These findings imply that YAP is critical for basal heart homeostasis, and YAP deficiency exacerbates myocardial apoptosis and fibrosis.

Studies in various cell lines or non-cardiac tissues consistently show that CTGF is up-regulated by YAP (Plouffe et al., 2016; L. Wang et al., 2016; J. Wang et al., 2018; F. X. Yu et al., 2012). We selected

CTGF, Birc5, and Ankrd1 as YAP-target genes (Chen et al., 2014; Kimura et al., 2016; Matsuda et al., 2016; Plouffe et al., 2016) and determined their expression. In hearts of isoprenaline-treated mice or Mst1-TG mice, however, expression of these genes were up-regulated, a finding that appears to indicate YAP activation. Considering our finding of up-regulated expression of Gal-3, the molecule possessing transcription activity in the heart (Rabinovich & Toscano, 2009; Suthahar et al., 2018), we elected to address a possibility that Gal-3 could regulate expression of these genes. We showed that Gal-3 gene deletion in both settings (i.e., isoprenaline treatment and Mst1-TG) significantly inhibited expression of these genes. This finding indicates that in the diseased heart, expression of some YAP-target genes might be indirectly regulated by Gal-3, while expression of Gal-3 per se is under tight control by the Hippo signalling. Thus, our results not only add Gal-3 and BIM to the list of YAP-target genes but also demonstrate regulation by Gal-3 of certain YAP-target genes.

In conclusion, we show that β -adrenoceptor stimulation results in PKA-dependent up-regulation of Mst1 and hyper-phosphorylation of YAP, indicating activation of the Hippo signalling pathway. Cardiac β -adrenoceptor stimulation leads to up-regulation of Gal-3 and BIM that is dependent on Mst1-activity and most likely due to YAP inactivation. β -Adrenoceptor antagonists effectively inhibited activation of the Hippo signalling pathway and expression of Gal-3 and BIM. This finding extends our understanding of the overall efficacy of β -blockers in heart disease. Our results imply that suppression of Gal-3 and BIM expression by targeting β -adrenoceptor-Mst1(Hippo) signalling could form a new therapeutic approach to the treatment of heart disease.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

W.B.Z., Q.L., M.N.N., and X.J.D. conceived and designed the experiments. Acquisition and analysis of data were performed by W.B.Z., M.N.N., Q.L., M.Z., Y.S., L.N.W., and H.K. Interpretation of data was carried out by W.B.Z., M.N.N., Q.L., M.Z., Y.S., L.N.W., H.P., J.S., H.Y.H., and X.J.D. W.B.Z. and X.J.D. drafted the manuscript. All authors revised the manuscript critically and approved the submission. Funds were obtained by X.J.D. and H.Y.H.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for [Design & Analysis, Immunoblotting and Immunochemistry](#), and [Animal Experimentation](#), and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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