Cardiovascular Research (2016) **109**, 358–373
doi:10.1093/cvr/cvv274

Bidirectional cross-regulation between ErbB2 and β-adrenergic signalling pathways

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Received 28 February 2014; revised 27 November 2015; accepted 1 December 2015; online publish-ahead-of-print 21 December 2015

Time for primary review: 32 days

Aims Despite the observation that ErbB2 regulates sensitivity of the heart to doxorubicin or ErbB2-targeted cancer therapies, mechanisms that regulate ErbB2 expression and activity have not been studied. Since isoproterenol up-regulates ErbB2 in kidney and salivary glands and β2AR and ErbB2 complex in brain and heart, we hypothesized that β-adrenergic receptors (AR) modulate ErbB2 signalling status.

Methods and results ErbB2 transfection of HEK293 cells up-regulates β2AR, and β2AR transfection of HEK293 up-regulates ErbB2. Interestingly, cardiomyocytes isolated from myocyte-specific ErbB2-overexpressing ($ErbB2^{tg}$) mice have amplified response to selective β2-agonist zinterol, and right ventricular trabeculae baseline force generation is markedly reduced with β2-antagonist ICI-118 551. Consistently, receptor binding assays and western blotting demonstrate that β2ARs levels are markedly increased in $ErbB2^{tg}$ myocardium and reduced by EGFR/ErbB2 inhibitor, lapatinib. Intriguingly, acute treatment of mice with β1- and β2-AR agonist isoproterenol resulted in myocardial ErbB2 increase, while inhibition with either β1- or β2-AR antagonist did not completely prevent isoproterenol-induced ErbB2 expression. Furthermore, inhibition of ErbB2 kinase predisposed mice hearts to injury from chronic isoproterenol treatment while significantly reducing isoproterenol-induced pAKT and pERK levels, suggesting ErbB2's role in transactivation in the heart.

Conclusion Our studies show that myocardial ErbB2 and βAR signalling are linked in a feedback loop with βAR activation leading to increased ErbB2 expression and activity, and increased ErbB2 activity regulating β2AR expression. Most importantly, ErbB2 kinase activity is crucial for cardioprotection in the setting of β-adrenergic stress, suggesting that this mechanism is important in the pathophysiology and treatment of cardiomyopathy induced by ErbB2-targeting antineoplastic drugs.

Keywords ErbB2 • ErbB2 kinase inhibitors • β-Adrenergic stimulation • β-Blockers

1. Introduction

ErbB2, a receptor tyrosine kinase (RTK) of the EGFR family, a co-receptor for EGFR, ErbB3, and ErbB4, is expressed in multiple cell types including cardiomyocytes. Among the possible ligand-receptor combinations,[1] the most studied in the heart is the ligand neuregulin 1β (NRG1β) which binds to ErbB4 on cardiomyocytes. NRG1β binding to ErbB4 promotes heterodimerization with ErbB2 leading to phosphorylation of the heterodimers and activation of downstream kinase pathways (ERK-MAPK and PI3K-Akt) affecting contractile function, proliferation, and cell survival. Importantly, myocyte-specific deletion of ErbB2 results in severe dilated cardiomyopathy[2,3] showing the significance of the ErbB2 pathway in cardiac remodelling.

ErbB2 is also expressed in breast epithelium and overexpressed in 25% of breast cancers. The importance of ErbB2 in the myocardium became clinically relevant when anti-ErbB2 treatment (Trastuzumab or Herceptin) was introduced into breast cancer treatment regimen. Approximately 27% of patients experienced cardiac dysfunction when

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anti-ErbB2 treatment was used in combination with the anthracycline doxorubicin,[4,5] a cytotoxic drug known to increase cardiac ErbB2 in patients,[6,7] and in animal models.[8] In the early stages of cardiac dysfunction, the protective NRG1β-ErbB2 axis is likely activated in myocardium,[9–11] but the mechanism of this is unknown.

Neurohormonal activity dynamically regulates cardiovascular function and may be a critical determinant of the status of the NRG/ErbB signalling axis. Previous studies have shown that (i) ErbB2 forms a complex with the protective G-protein coupled receptor (GPCR) β2-adrenergic receptor (β2AR) critically regulating downstream ERK1/2 activation[12] via transactivation, (ii) ErbB2 and β2AR are linked in a feedback loop in cancer cells,[13] and (iii) ErbB2 is increased in salivary gland[14] and kidney[15] after β-adrenergic stimulation with isoproterenol. Based on these salient observations, we hypothesized that ErbB2 and βAR signalling pathways are connected in the heart through a bidirectional cross-regulation feedback loop wherein inhibition of the ErbB2 pathway disrupts this protective loop.

In our current study, we determined that (i) ErbB2 protein levels are increased in the heart during acute βAR stimulation, (ii) ErbB2 up-regulation following β-adrenergic stimulation is dependent on β1 and/or β2 adrenergic receptor, (iii) ErbB2 up-regulates β2AR in the heart and *in vitro* systems, (iv) β2AR up-regulates ErbB2 in *in vitro* systems, (v) ErbB2 inhibitors abolish β2AR up-regulation and protein expression involved in the protective feedback loop, (vi) β-blockers reduce the size of the heart in the ErbB2^{tg} mice, (vii) ErbB2 inhibitors reduce the size of the heart in wild-type mice, (viii) isoproterenol induces an increase in cleaved products of ErbB4 and neuregulin indicative of pathway activity, (ix) ErbB2 kinase inhibitors reduce isoproterenol-induced pAKT and pERK in the mouse heart, and (x) ErbB2 kinase is protective during chronic treatment with isoproterenol. These findings support a bidirectional connectivity between ErbB2 and βAR signalling pathways and/or dependence on ErbB2 in the myocardium during β-adrenergic stress. Thus, our study is interesting in reference to the recent epidemiological studies where Trastuzumab toxicity was reduced in women given β-blockers[16,17] suggesting further studies are needed to better understand the protective relationship of ErbB2 in conditions of β-adrenergic stress in the heart.

2. Methods

Reagents and drugs used in the experiments are listed in Supplementary material online, *Table S1*.

2.1 Animal studies

This study was performed in accordance with the 'Guide for the Care and Use of Laboratory Animals' (2011) of the National Institutes of Health. The protocol was approved by the Animal Care and Use Committee of the Johns Hopkins Medical Institutions (Animal Welfare Assurance no. A-3273-01). Eight- to ten-week-old female mice (average body weight 18–25 g) were used for the *in vivo* experiments. All mice were housed under a 12 h light–dark cycle with free access to food and water. Euthanasia was performed using carbon dioxide (CO_2), according to AVMA Guidelines for the Euthanasia of Animals (June 2007) and Johns Hopkins University Animal Care and Use Committee Guidelines for Euthanasia of Rats and Mice Using Carbon Dioxide (July 2008). After euthanasia, the mice were weighed, the hearts were excised, weighed, and cut transversely; left ventricle, right ventricle, and septum were immediately frozen for further molecular studies. Left ventricle was used in all the molecular studies presented here.

2.2 *In vitro* transfection studies

HEK293 were obtained from ATCC and maintained in DMEM supplemented with 10% FBS. Cells were transfected with rat-neu/ErbB2 or the pcDNA 3.1 (+) control plasmid using Lipofectamine reagent. After 48 h, the cells were washed with cold 1× PBS and harvested in 1× PBS with a cell scraper and centrifuged. HEK 293 cells were also transfected with human β2AR expressing cDNA in pcDNA 3.1 (+). The transfected cells were selected on G418 for neomycin resistance. G418 selection resulted in generation of HEK 293 cells stably expressing human β2AR. As a first confirmatory step to determine stable expression of human β2ARs in HEK 293 cells, plasma membranes were isolated and radio ligand binding was performed as described in the receptor binding method section below. Once β2AR expression was confirmed, the cells were scraped in 1× PBS and centrifuged. Lysis buffer was added to the cell pellet and spun down. The final supernatant contained the soluble protein used for western blotting.

2.3 Transgenic model

The cardiac-specific ErbB2^{tg} mouse model was generated as described.[18] Eight- to ten-week-old female WT and ErbB2^{tg} mice were used for all studies, unless otherwise indicated.

2.4 *In vivo* β-agonists and β-blockers treatments

(−)-Isoproterenol hydrochloride was diluted in 0.9% saline and administered via intraperitoneal injection (1 mg/kg) to B6SJLF1/J mice. Saline control mice were injected with a comparable volume of 0.9% saline. All mice were euthanized at 30 min[19] in acute and chronic studies to compare signalling pathway activation across the treatment groups. Quickly, the hearts were collected, sectioned, and frozen. In separate experiments, the mice were injected with 1 mg/kg of isoproterenol and euthanized 24 h later. Metoprolol tartrate was dissolved in drinking water (0.45 g/L), so that the mice would receive in average of 90 mg/kg/day.[20] The control group received regular drinking water in similar water bottles. Mice were administered metoprolol for 5 days, and on Day 5, they were injected with saline or isoproterenol (1 mg/kg). ICI 118 551 (4 mg/kg)[21] was diluted in 0.9% saline and administered to mice intraperitoneally, and 1 h later, mice were injected with saline or isoproterenol (1 mg/kg). The injectable drugs concentrations were adjusted so that animals received similar volumes of the diluted drug and the vehicle. These studies were performed in the animal facility procedures rooms between 2 and 4 pm to reduce study variability.

To explore the effects of β-blockers on the heart weights in WT and ErbB2^{tg} mice, genotype combined litters ($n = 12$ litters) of WT and ErbB2^{tg} mice littermates were exposed to regular water, metoprolol tartrate, or propranolol hydrochloride from Day 1 to Day 55. Dams were given treated water when pups were born (Day 1), and mice were exposed via either milk or drinking water (as soon as they started using drinking bottles) until euthanasia (Day 55). Metoprolol was dissolved in drinking water at 0.45 g/L so that the mice would receive in average of 90 mg/kg/day.[18] Amber bottles were used to protect light-sensitive metoprolol solution. Propranolol was dissolved at 0.5 g/L.[22] Bottles were cleaned and refilled twice a week. Availability of drug in milk was not tested, yet with weaning at 3 weeks, mice did continuously receive treated water. The control litters received regular drinking water in similar water bottles. At euthanasia, mice and hearts were weighed, and tibia lengths were measured.

2.5 Adult cardiomyocyte isolation and functional parameter measurements

Cardiomyocytes were isolated from at least three mice per genotype and per treatment group on at least three separate days. For some of the experiments, particularly for ErbB2^{tg} mice, four or five hearts were used, because ErbB2^{tg} mice generally had lower cell yields. The specific animal numbers for each particular experiment are given in the figure legends.

Hearts were quickly removed from the chest after euthanasia, and aorta was retroperfused at 100 cm H_2O and 37°C for ~3 min with a Ca^{2+}-free

bicarbonate-based buffer (see Supplementary material online, *Table S2*), gassed with 95% O_2–5% CO_2. Enzymatic digestion was initiated by addition of 0.9 mg/mL collagenase type 2 (299 U/mg) and 0.05 mg/mL protease type XIV to the perfusion solution (6–7 min). Dispersed cardiomyocytes were filtered through a 150 μm mesh and gently centrifuged at 500 rpm for 30 s. The pellet was re-suspended in Tyrode's solution with increasing Ca^{2+} (1 mM), and cells were then incubated for 10 min with 3 μmol/L Fura 2-AM in Tyrode's solution (1 mM Ca^{2+}). After rinsing, cells were placed in a perfusion chamber with a flow-through rate of 2 mL/min, and functional measurements were made: sarcomere shortening, whole-cell Ca^{2+} transients, relaxation, and Ca^{2+} reuptake times. The functional parameters were measured at the baseline and with isoproterenol (10 nM), zinterol (1 μM),[23] or forskolin (100 nM), using an inverted fluorescence microscope (Nikon, TE2000) and IonOptix (Myocam®) software.[24]

2.6 Trabeculae isolation and systolic force measurements

Trabeculae from right ventricles ($n = 4$ WT or ErbB2^{tg} mice) were dissected in Krebs–Henseleit (K-H) solution (1 mM Ca^{2+}) (see Supplementary material online, *Table S2*). The muscle was mounted between a force transducer and a motor arm, superfused with K-H solution at room temperature (10 mL/min) and stimulated at 0.5 Hz. Force was measured using a force transducer and was expressed in mN/mm^2. The muscles underwent isometric contractions with the resting muscle length set such that resting force was 15% of total force development (i.e. optimal muscle length). This resting muscle length corresponds to a resting sarcomere length of 2.20–2.30 μm as determined by laser diffraction.[25] The measurements were repeated with ICI 118 551 (1 μM).

2.7 β-Adrenergic receptor binding assay

Radio ligand βAR binding studies were carried out as previously described.[26] Briefly, hearts ($n = 5$ mice per group) were homogenized in lysis buffer (5 mmol/L Tris–HCl pH 7.5, 5 mM EDTA, 1 mM PMSF, and 2 μg/mL Leupeptin and Aprotinin). The homogenized samples were centrifuged at 1000 *g* for 5 min to remove cell debris/nuclei and the supernatant was centrifuged at 37 000 *g* for 20 min. Pellet representing membrane fraction was re-suspended in 75 mM Tris–HCl pH 7.5, 2 mM EDTA, and 12.5 mM $MgCl_2$. βAR density was determined by incubating 25 μg of the membranes with saturating concentrations of ^{125}I Cyanopindolol along with 40 μM propranolol for non-specific binding as previously described.[27] To assess β1/β2AR ratios, 40 μM metoprolol and/or 100 μM ICI 118 551[28] were used.

2.8 cAMP measurements

Cells were harvested in NP40 lysis buffer containing 20 mM Tris pH 7.4, 137 mM NaCl, 1% NP-40, 1 mM PMSF, 20% glycerol, 10 mM NaF, 1 mM sodium orthovanadate, 2 mg/mL Leupeptin, and Aprotinin. The lysates were cleared by centrifugation at 12 000 *g* for 15 min at 4°C. The supernatants were used for determining the cAMP content[29] using catch point cAMP kit (Molecular Devices, Sunnyvale, CA, USA) as per manufacturer's instruction.

2.9 Western blotting

Left ventricle lysates were made from the hearts of 8- to 10-week-old WT or ErbB2^{tg} female mice. Frozen tissue was rapidly homogenized in 200–300 μL of lysis buffer (see Supplementary material online, *Table S2*), and standard gel electrophoresis and western blotting were performed. Four to 12% NuPage Bis–Tris gels (Life Technologies, Grand Island, NY, USA) were used, except for phospholamban evaluation where 13% Tris–Glycine gels were made. The antibodies used are listed in Supplementary material online, *Table S3*. After incubation in anti-rabbit or anti-mouse (1:5000; GE Healthcare, Piscataway, NJ, USA) or anti-chicken (1:20 000), horseradish peroxidase-linked secondary antibody, chemiluminescent substrate was applied (Pierce, Rockford, IL, USA; GE Healthcare, Piscataway, NJ, USA), and membranes were exposed to CL-Xposure film (Pierce, Rockford, IL, USA) or Blu-Ray Film (NextDayScience, Rockville, MD, USA). AKT protein levels were used as loading controls, as previously used by others,[30–32] as AKT was preferable since actin and GAPDH both varied comparing WT or ErbB2^{tg} mice. Densitometry was performed using ImageJ (NIH software). Representative images are shown and all the images within comparison groups are taken from the same membrane (same exposure).

2.10 Real-time PCR

Total RNA was isolated from the left ventricle of (8–10 weeks old, 3 mice per group) using RNeasy Mini Kit (Cat. no. 74104, Qiagen, Valencia, CA, USA), with an in-column DNase treatment (RNase-Free DNase Set, Qiagen) as described.[8] cDNA synthesis was performed using a SuperScript II kit (Invitrogen). cDNA was used to evaluate ErbB2 and RCAN1 mRNA levels by quantitative real-time reverse transcriptase–PCR (qRT–PCR) using iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). TaqMan® (Applied Biosystems, Foster City, CA, USA) assay (mm00658541_m1) was used for ErbB2 mRNA levels evaluation and the following primers (Integrated DNA Technologies, Coralville, IA, USA) were used for RCAN1 mRNA levels evaluation: 5′-gagtcgttcgttaagcgtc-3′ (F) and 5′-aaatttggccctggtctcac-3′ (R). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or peptidylprolyl isomerase A (PPIA) mRNA levels were used for RNA normalization. The quantitative RT–PCR results were calculated by ΔΔCt method using the mean of the ΔCt value of vehicle-treated mice as a normalization factor.

2.11 Lapatinib treatment

WT and ErbB2^{tg} mice were treated with oral lapatinib (160 mg/kg/day) ($n = 7$ mice per group), via a 20G-38 mm gavage needle (Harvard Apparatus, Holliston, MA, USA). A suspension of lapatinib (LC Laboratories, Woburn, MA, USA) was freshly prepared before each treatment, by diluting lapatinib with a vehicle buffer containing 0.5% carboxymethylcellulose, 1.8% sodium chloride, and 0.4% Tween 80 in dH_2O. Appropriate volumes of the vehicle were administered to control mice. Since a percentage of lapatinib is excreted in faeces, vehicle- and lapatinib-treated mice were housed separately. The mice were treated daily for 11 days, euthanized 2 h after receiving the final treatment (at peak serum levels), and the hearts were collected.

2.12 AG825 and isoproterenol studies

Female B6SJLF1/J mice ($n = 5$ per group) were randomly divided into five treatment groups: saline, DMSO, AG825 (ErbB2 kinase inhibitor), isoproterenol, and DMSO or isoproterenol and AG825. All treatments were given twice per day in the morning and evening, ~12 h apart for 2 weeks. Mice were injected with isoproterenol (7.5 mg/kg) each injection. Saline and isoproterenol were injected subcutaneously between shoulder blades. DMSO (0.01%) and AG825 (1 mg/kg) (dissolved in 0.01% DMSO) were injected into right or left flank subcutaneous tissue. Transthoracic echocardiography was performed on Day 7 and Day 14 to measure systolic function. One hour after echocardiography evaluation, mice were given the final injections and euthanized 30 min later. We chose this time point to evaluate isoproterenol-induced ERK and AKT phosphorylation. Heart weights/tibia lengths were measured at euthanasia. Representative cross-sections of hearts were saved for histology, processed, and later stained with fibrosis stain Masson's Trichrome. The remainder of left ventricle was sectioned in strips from base to apex and frozen in liquid nitrogen, and lysates were made from these sections.

2.13 Echocardiography

Transthoracic echocardiography was performed on conscious mice using Acuson Sequoia C256 ultrasound machine equipped with the 15 MHz linear array transducer. The mouse heart was imaged in a two-dimensional

mode followed by M-mode using the parasternal short-axis view at a sweep speed of 200 mm/s. Measurements were acquired using the leading edge method, according to the American Echocardiography Society guidelines.[33] Left ventricle chamber dimensions were acquired during the end-diastolic and end-systolic phase, including interventricular septum (IVSD), left ventricular posterior wall thickness (PWTED), left ventricular end-diastolic dimension (LVEDD), and left ventricular end-systolic dimension (LVESD). Three to five values for each measurement were acquired and averaged for evaluation. The LVEDD and LVESD were used to derive fractional shortening (FS) and to measure left ventricular performance by the following equation: FS% = [(LVEDD − LVESD)/LVEDD] × 100.

2.14 Statistics

For statistical analysis, GraphPad Prism software (GraphPad, La Jolla, CA, USA) was used. Data are presented as mean + SD. The unpaired Student's *t*-test or analysis of variance (ANOVA) were performed to compare two or three or more unrelated groups as appropriate, with a *P*-value of <0.05 deemed significant.

3. Results

3.1 Erbb2 overexpression up-regulates β2AR, while β2AR overexpression up-regulates ErbB2 *in vitro*

The previous work on the cooperative relationship between β2AR and ErbB2 led to our investigations on whether elevation in ErbB2 protein itself would increase endogenous β2AR protein levels, or vice versa, whether an increase in β2AR protein in turn would increase ErbB2 protein levels. In this context, it is known that ErbB2 and β2AR co-localize in lipid rafts[34,35] and both receptors co-immunoprecipitate in a complex,[12] but it is not known whether these two receptors cross-regulate the protein expression of their respective partners. To examine this possibility, HEK293 cells were transiently transfected with a cDNA construct encoding ErbB2. Remarkably, endogenous β2-AR was up-regulated with ErbB2 overexpression (*Figure 1A*). A concurrent increase in AKT phosphorylation was also found in these cells demonstrating pathway activation (*Figure 1A*).

Since transient expression of ErbB2 in HEK293 cells resulted in marked increase in endogenous β2AR expression, we next tested whether overexpression of β2AR in HEK 293 cells would result in a counter regulation of ErbB2. Intriguingly, we observed a marked increase in ErbB2 expression in the β2AR-overexpressing cells (*Figure 1B*). To assess whether overexpression of β2AR, which in parallel up-regulates endogenous ErbB2, has consequences in downstream signals at baseline, immunoblotting was performed for phospho-ERK and phospho-Akt. A minimal increase in phospho-ERK was observed with no changes in phospho-Akt (data not shown). To further provide evidence of β2AR overexpression in HEK293 cells, plasma membranes from these cells were subjected to $^{[125]}$I-cyanopindalol radio ligand binding. Significant expression of β2ARs was observed in HEK293 cells expressing β2AR compared with control vector transfected cells (*Figure 1C*). Consistently, cAMP was significantly increased at baseline (with no added agonist stimulation) in β2AR-expressing cells compared with vector controls (*Figure 1D*).

3.2 Erbb2 increases NRG1β (neuregulin), ErbB4 and its cleaved products, phospholamban (PLN) levels and eNOS phosphorylation, calcium transients, and SR Ca^{2+} load in isolated cardiomyocytes or myocardium from ErbB2^{tg} mice compared with WT littermates

To evaluate the physiologic role of ErbB2 and β2AR signalling in the myocardium, we isolated adult cardiomyocytes from transgenic mice with myocyte-specific expression of ErbB2 (ErbB2^{tg}) to compare baseline calcium cycling and sarcomere shortening. ErbB2^{tg} mice have concentric hypertrophy but do not progress to end-stage heart failure.[18] Adult ErbB2^{tg} cardiomyocytes had significantly increased Ca^{2+} transients accompanied by faster sarcoplasmic reticulum (SR) Ca^{2+} reuptake (*Figure 2A*; *Table 1*) and elevated SR Ca^{2+} load (*Figure 2B* and *C*) compared with WT cardiomyocytes. Interestingly, basal sarcomere shortening (SS, %) of adult ErbB2^{tg} cardiomyocytes was similar to WT cardiomyocytes in spite of higher Ca^{2+} transient and faster relaxation in ErbB2^{tg} cardiomyocytes (*Figure 2A*; *Table 1*). This observation is reminiscent of the negative inotropic effects observed previously with ErbB4 ligand NRG1β.[36,37]

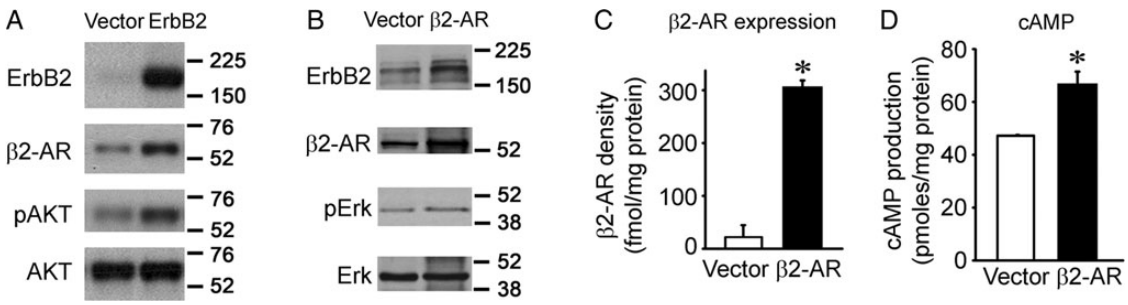

Figure 1 *In vitro* ErbB2 overexpression causes β2AR up-regulation and AKT phosphorylation, while β2AR overexpression causes ErbB2 up-regulation. (*A*) HEK293 cells were transfected with ErbB2, and 48 h later the cells were harvested, lysates were prepared, and western blotting was performed. ErbB2 transfected cells have a concurrent up-regulation of β2-adrenergic receptor and AKT phosphorylation. (*B*) Protein levels of ErbB2, β2AR, pErk1/2, Erk1/2 were evaluated by western blotting in the lysates made of HEK 293 cells transfected with empty vector or β2AR expression vector. (*C*) Radio ligand binding assay was performed using $^{[125]}$I cyanopindolol methods in plasma membranes of HEK 293 cells expressing β2ARs or vector, ($n = 4$). (*D*) Baseline cAMP levels were measured in HEK 293 cells expressing β2ARs or vector ($n = 3$). Data are in means ± SD (*B*) $^*P < 0.001$, (*D*) $^*P < 0.01$ by Student's unpaired *t*-test.

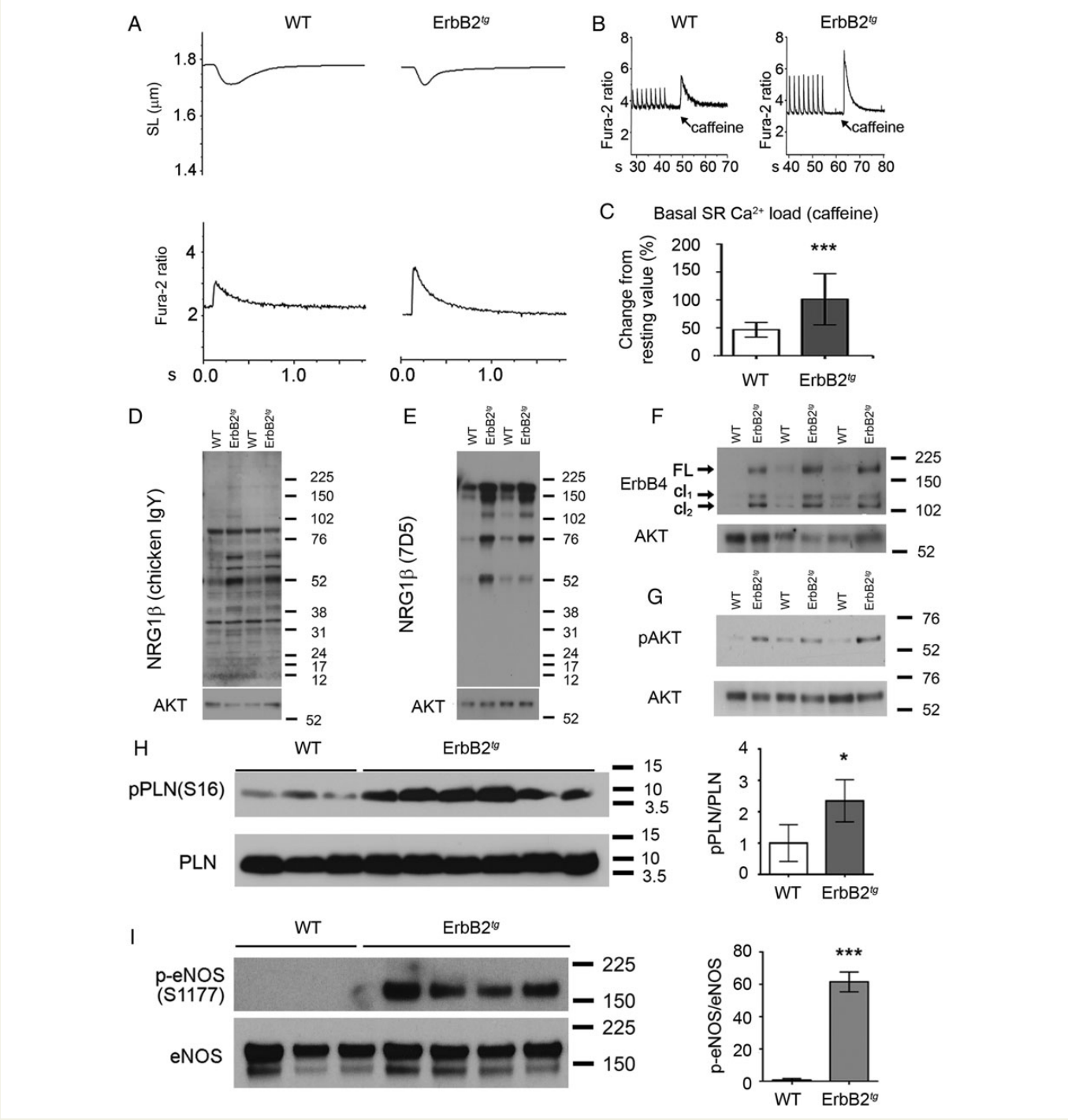

Figure 2 ErbB2 overexpression effects on calcium handling in murine isolated cardiomyocytes, phosphorylation of phospholamban (PLN), and RCAN1 expression. (*A*) Representative tracings of isolated murine wild-type (WT) and ErbB2-overexpressing (ErbB2^{tg}) adult cardiomyocytes showing the effects of ErbB2 overexpression on sarcomere shortening (SL = sarcomere length, μm) and whole-cell Ca^{2+} transients (Fura-2 fluorescence ratio). Cardiomyocytes were isolated from four mice per genotype on at least three separate days. (*B* and *C*) Effects of ErbB2 overexpression on caffeine-induced sarcoplasmic reticulum (SR) Ca^{2+} transient: representative tracings (*B*), and quantification (*C*); $n = 26$ cells (WT), 10 cells (ErbB2^{tg}) (all cells derived from four successful isolations). $^{***}P < 0.001$ by the Student unpaired *t*-test. The data are means ± SD. (*D* and *E*) Neuregulin 1β (NRG-1β) (*D* and *E*), ErbB4 (*F*) expression, and phospho-AKT (*G*) levels were evaluated in the left ventricles of the WT and ErbB2^{tg} mice by western blotting. (*H*) PLN phosphorylation (Serine 16) in the hearts of WT and ErbB2^{tg} mice was evaluated by western blotting and normalized to total PLN. Representative images (left) and densitometric quantifications (fold change from WT, right) are shown. $n = 3$ (WT), $n = 6$ (ErbB2^{tg}) mice per group. $^{*}P < 0.05$ by the Student unpaired *t*-test. The data are means ± SD. (*I*) eNOS phosphorylation (Serine 1177) levels in the hearts of WT and ErbB2^{tg} mice were evaluated by western blotting and normalized to total eNOS protein levels. Representative images (top) and densitometric quantifications (fold change from WT, bottom) are shown, $n = 3$ (WT), $n = 4$ (ErbB2^{tg}). $^{*}P < 0.05$, $^{***}P < 0.001$ by the Student unpaired *t*-test. The data are the means ± SD.

Table 1 Isolated murine cardiomyocytes baseline data

	WT	ErbB2^{tg}	*P*-value
SS, %	3.62 ± 1.13	3.76 ± 1.11	0.6113
Ca^{2+} transient, %	38.73 ± 15.11	69.7 ± 27.81	<0.0001***
RT_{50}, ms	402.7 ± 130	292 ± 80	0.0001***
Ca^{2+} transient RT_{50}, ms	221.18 ± 70	183.79 ± 40	0.0087**

Cardiomyocytes were isolated from five mice per genotype on at least 3 separate days. Sarcomere shortening [SS, %, $n = 38$ cells (WT), $n = 27$ cells (ErbB2^{tg})], Ca^{2+} transient [%, $n = 36$ cells (WT), $n = 26$ cells (ErbB2^{tg})], RT_{50} [ms, $n = 40$ cells (WT), $n = 28$ cells (ErbB2^{tg})], and calcium transient RT_{50} [ms, $n = 38$ cells (WT), $n = 28$ cells (ErbB2^{tg})] (all cells derived from five successful isolations) of isolated murine WT and ErbB2^{tg} cardiomyocytes. **$P < 0.01$, ***$P < 0.001$ by the Student unpaired *t*-test. The data are the means ± SD.

We have previously reported that pAKT and pERK were elevated in the ErbB2^{tg} mice[18] but had not determined whether any ErbB pathway ligands were elevated in myocardium. Since the overexpression of ErbB2 also induces elevation of multiple proteins in its pathway,[18] we next assessed for ErbB4 ligand NRG1β in the transgenic ErbB2^{tg} heart. Measurement of NRG1β in the myocardium by western blotting showed that membrane and cleaved forms of NRG1β were increased in ErbB2^{tg} compared with WT littermates (*Figure 2D* and *E*). Since recent studies have shown that NRG1β ligand activity leads to generation of cleaved ErbB4 products,[38] we examined expression of ErbB4 by immunoblot. Myocardial lysates from ErbB2^{tg} mice showed significant increase in full length and cleaved products of ErbB4 (*Figure 2F*). Corresponding to the heterodimerization of ErbB2 and ErbB4 in the context of elevated NRG1β, we observed significant activation of downstream kinase Akt (*Figure 2G*).

Since calcium reuptake was increased in isolated ErbB2^{tg} cardiomyocytes, we tested for the underlying mechanism by assessing activation status of phospholamban and eNOS. Phospholamban phosphorylation (S16) was significantly increased in ErbB2^{tg} hearts (*Figure 2H*), suggesting that faster SR Ca^{2+} uptake is related to reduction of SERCA inhibition through PLN phosphorylation. Furthermore, ErbB2^{tg} mice myocardium is associated with increased phosphorylated eNOS levels (S1177) (*Figure 2I*) compared with WT. This is interesting as activated eNOS has been implicated in the negative inotropic effect of NRG1β treatment.[36,37] We therefore compared our findings in ErbB2^{tg} mice with various models of NRG1β treatment[36,37,39,40] to show the similarities and differences (*Table 2*). Activation of the eNOS pathway, likely due to activation by pAKT, appears to be a common link between our studies and the previous work by others using the ErbB4 ligand NRG1β.

3.3 Erbb2^{tg} cardiomyocytes and trabeculae have increased β2AR levels and physiological response

ErbB2^{tg} cardiomyocytes treated with the β2-adrenergic receptor agonist zinterol exhibited an amplified positive inotropic response that matches the inotropic effect of the non-selective βAR agonist isoproterenol (*Figure 3A* and *B*). ErbB2^{tg} cardiomyocytes treated with forskolin (direct adenylyl cyclase activator) also had a similar increase in sarcomere shortening and whole calcium transients compared with WT cardiomyocytes (*Figure 3C*) suggesting that the molecular difference in pathway response between WT and ErbB2^{tg} mice lies in the proximal β2AR signalling pathway. In support, ^{125}I-cyanopindolol radio ligand binding showed that β2AR density was doubled in ErbB2^{tg} myocardium (*Figure 3D*), an observation consistent with the *in vitro* HEK293 studies showing up-regulation of endogenous β2ARs with ErbB2 expression. Corresponding to the myocyte studies, loaded ErbB2^{tg} trabeculae had significantly higher baseline force (*Figure 3E*). Importantly, the baseline force was significantly reduced (compared with the WT trabeculae) under exposure to selective β2AR inhibitor ICI 118 551 (*Figure 3E*). In summary, both cardiomyocyte and trabeculae studies suggest that the higher level of β2AR expression may underlie the observed myocardial responses in the ErbB2^{tg} mice.

Table 2 Functional comparison of ErbB2 overexpression (current study) and NRG1β treatment (published in literature) in isolated cardiomyocytes, trabeculae (or papillary muscles), or hearts *in vivo*

Parameter values or change of parameters (compared to WT or untreated control)	ErbB2^{tg} vs. WT (current study)	NRG treated vs. untreated (published in literature)
Isolated cardiomyocytes, baseline		
1 Sarcomere shortening, %	Unchanged	Decreased[40]
2 Ca^{2+} transient	Increased	Unchanged[40] or increased[36]
3 Relaxation	Faster	Unchanged[40]
4 Ca^{2+} reuptake	Faster	Faster[36]
Isolated cardiomyocytes, ISO-induced changes, compared with changes in respective controls		
5 Sarcomere shortening, %	Decreased	–
6 Ca^{2+} transient	Unchanged	Unchanged[36]
7 Relaxation	Faster	–
8 Ca^{2+} reuptake	Unchanged	–
Caffeine-induced Ca^{2+} transients in isolated cardiomyocytes		
9 Ca^{2+} transient	Increased	Unchanged[40] or Increased[36]
Trabeculae or papillary muscles		
10 Force (active tension)	Increased	Decreased[37]
Signal transduction		
11 Phospholamban phosphorylation (Ser16)	Increased	Increased[36] or Decreased[39]
12 eNOS phosphorylation (Ser1177)	Increased	Increased[36,37]

(1–9) Functional changes comparison between isolated murine cardiac ErbB2^{tg} cardiomyocytes (current study) and NRG1β-treated WT adult rat cardiomyocytes (literature search); (10) Functional changes comparison between isolated murine cardiac ErbB2^{tg} trabeculae (current study) and NRG1β-treated WT papillary muscles (literature search); (11) Phospholamban phosphorylation changes comparison between left ventricles obtained from ErbB2^{tg} (current study) and NRG1β-treated isolated WT adult rat cardiomyocytes or murine hearts (literature search); (12) eNOS phosphorylation changes comparison between left ventricles obtained from ErbB2^{tg} (current study) and NRG1β-treated isolated WT adult and neonatal rat cardiomyocytes (literature search).

3.4 β2AR is up-regulated in ErbB2^{tg} myocardium and ErbB2/EGFR kinase inhibitor lapatinib reduces β2AR expression

Since we observed receptor binding assays and physiological responses with agonists and antagonists that suggested higher β2ARs in

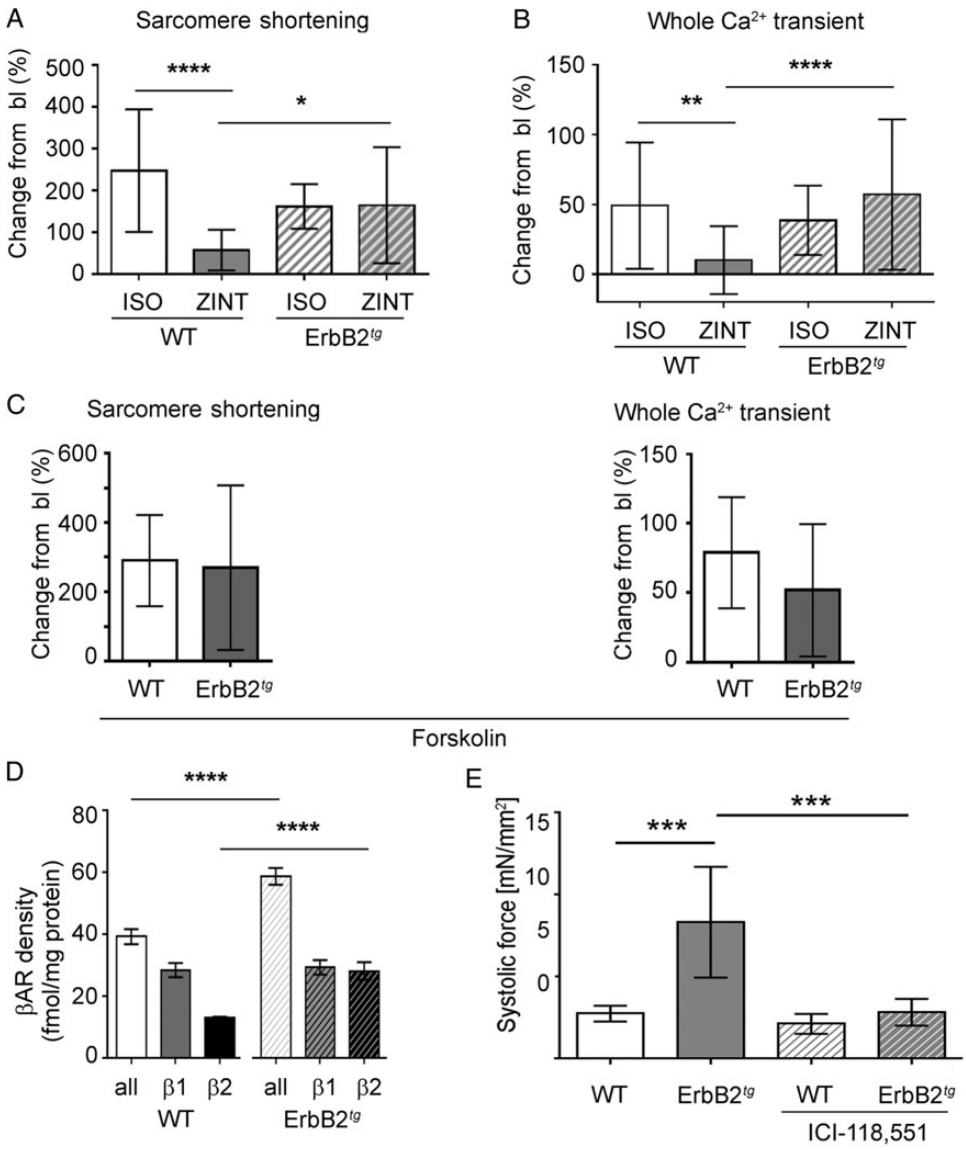

Figure 3 ErbB2 overexpression modulates the response of murine isolated cardiomyocytes to βAR stimulation. (*A* and *B*) Cardiomyocytes were isolated from at least three mice per genotype and per treatment group on at least three separate days. The β2-specific agonist zinterol (1 μM) induces an increase in sarcomere shortening and Ca^{2+} transients that matches the response to isoproterenol (10 nM, β1 and β2 agonist) in ErbB2^{tg} cells. In WT cells, zinterol is less potent than isoproterenol. Data are presented as percentage change from the baseline. Sarcomere shortening data: $n = 29$ cells (WT, ISO), $n = 17$ cells (ErbB2^{tg}, ISO); $n = 24$ cells (WT, Zinterol), $n = 18$ cells (ErbB2^{tg}, Zinterol). Ca^{2+} transient data: $n = 27$ cells (WT, ISO), $n = 16$ cells (ErbB2^{tg}, ISO); $n = 23$ cells (WT, Zinterol), $n = 17$ cells (ErbB2^{tg}, Zinterol) [all cells derived from four (WT) or five (ErbB2^{tg}) successful isolations]. $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$ by two-way ANOVA with *post hoc* Sidak's multiple comparisons test. The data are means ± SD. (*C*) The direct adenylyl cyclase stimulator forskolin (100 nM) elicits a similar response both in WT and in ErbB2^{tg} cardiomyocytes. Data are presented as percentage change from the baseline. Sarcomere shortening data: $n = 7$ cells (WT), $n = 12$ cells (ErbB2^{tg}). Ca^{2+} transient data: $n = 7$ cells (WT), $n = 13$ cells (ErbB2^{tg}) [all cells derived from three (WT) or four (ErbB2^{tg}) successful isolations]. The Student unpaired *t*-test was used for statistical analysis. The data are means ± SD. (*D*) Radioligand binding assays demonstrate a doubling of β2AR density in ErbB2^{tg} mice myocardium. $n = 5$ mice per group. $****P < 0.0001$ by one-way ANOVA with *post hoc* Tukey's test. The data are means ± SD. (*E*) Loaded right ventricular trabeculae of ErbB2^{tg} mice demonstrate increased baseline force generation which is normalized to normal levels by the β2-specific antagonist ICI 118 551 (1 μM). $n = 6$ (WT), $n = 5$ (ErbB2^{tg}) mice per group. $***P < 0.001$, $****P < 0.0001$ by two-way ANOVA with *post hoc* Sidak's multiple comparisons test. Data are means ± SD.

the myocardium of ErbB2^{tg} mice, we explored whether the expression of β2ARs is regulated by ErbB2/EGFR signalling by using lapatinib (ErbB2/EGFR kinase inhibitor) daily for 11 days. Consistent with our binding studies, β2AR was elevated in the myocardium of ErbB2^{tg} compared with WT littermate controls and lapatinib treatment reduced the levels of β2-AR protein in ErbB2^{tg} mice (*Figure 4A*). These studies confirm the role of EGFR/ErbB2 kinases in regulating the expression of β2-AR protein in the heart.

As high Ca^{2+} transients is observed with ErbB2^{tg} cardiomyocytes, we investigated the expression of RCAN1, a protective inhibitory protein of calcineurin-NFAT pathway that is responsive to calcium levels.[41,42] Indeed, increased expression of both RCAN1 mRNA

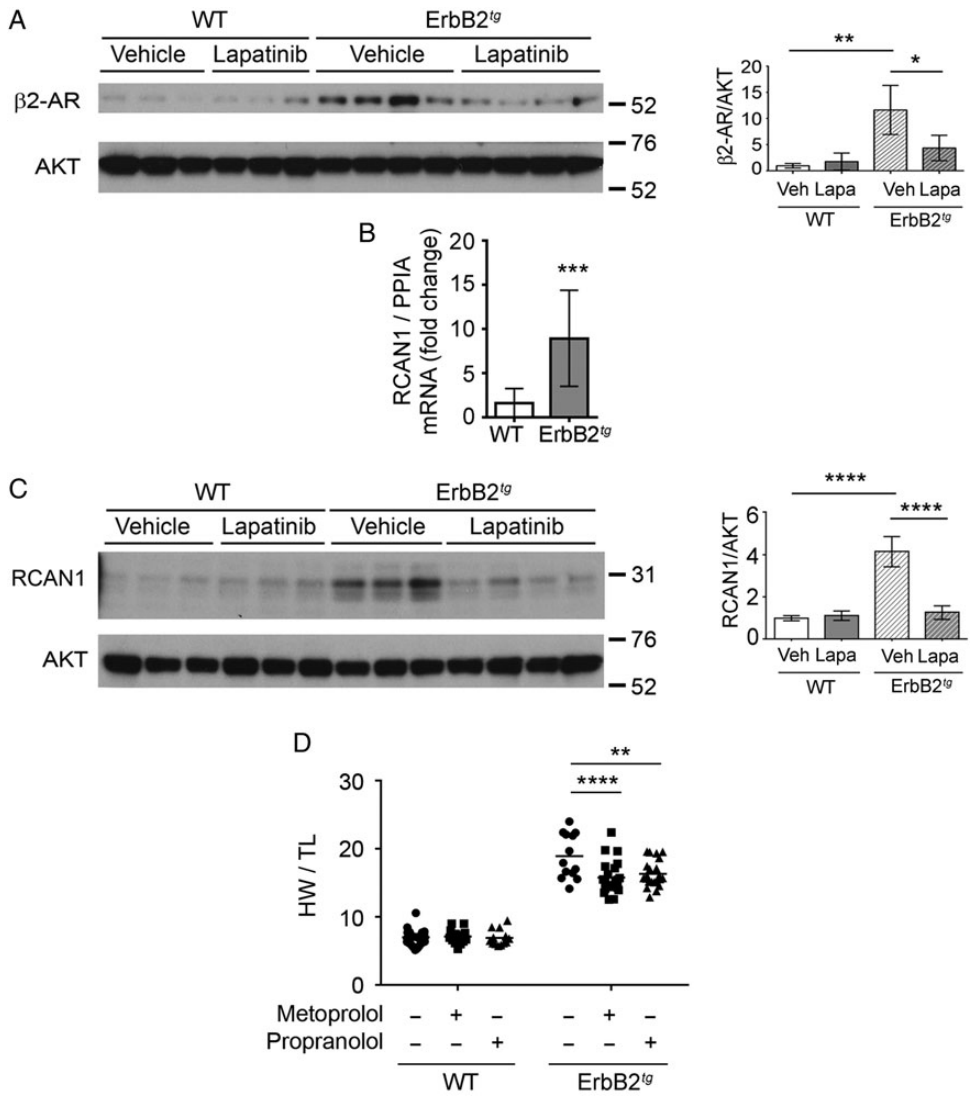

Figure 4 ErbB2 overexpression induces an increase in β2AR and RCAN1 protein in myocardium and reversed by lapatinib, ErbB2, and EGFR kinase inhibitor, while β-blockers reduce heart weights of ErbB2^{tg} mice. (*A*) β2AR and (*C*) RCAN1 protein in WT and ErbB2^{tg} myocardium (vehicle or lapatinib-treated) evaluated by western blotting and normalized to AKT protein levels. Representative images (left) and densitometric quantifications (right) are shown. Adult male WT and ErbB2^{tg} mice were given lapatinib (160 mg/kg of body weight) or vehicle (Veh) by gavage daily for 11 days. Two hours after the last lapatinib administration (peak serum levels), the mice were euthanized; the hearts excised, and western blotting of left ventricular lysates was performed. (β2AR or RCAN1; WT control, WT LAPA; ErbB2^{tg} control, ErbB2^{tg} LAPA). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$ by two-way ANOVA with *post hoc* Sidak's multiple comparisons test. The data are means ± SD. (*B*) Regulator of Calcineurin 1 (RCAN1) transcript expression levels by RT–PCR is increased in ErbB2^{tg} mice. Peptidylprolyl isomerase A (PPIA) was used as internal control. $n = 11$ (WT), $n = 12$ (ErbB2^{tg}), $n = 6$ WT LAPA, $n = 6$ ErbB2^{tg} LAPA mice/group, $^{***}P < 0.001$ by the Student unpaired *t*-test. The data are means ± SD. (*D*) Chronic treatment with metoprolol (0.45 g/L) or propranolol (0.5 g/L) delivered in the drinking water was performed in neonatal mice beginning Day 1. Fifty-five days later, the mice were euthanized and hearts were weighed and indexed by tibia lengths. $^{**}P < 0.01$, $^{****}P < 0.0001$ by two-way ANOVA with *post hoc* Sidak's multiple comparisons test. The data are means ± SD.

(*Figure 4B*) and RCAN1 protein was observed in ErbB2^{tg} hearts compared with WT hearts. Lapatinib treatment also decreased RCAN1 protein expression indicative of EGFR/ErbB2 kinase regulatory involvement maintaining RCAN1 protein level in the heart (*Figure 4C*).

To examine whether the heart size in the ErbB2^{tg} mice is reduced by β-blockers, neonatal mice were treated with β-blockers and the heart weights to tibia lengths compared across groups. Exposure of the mice to β-blockers (a non-specific and a β1-specific blocker) prior to development of cardiac hypertrophy/cardiomegaly, starting at postnatal day 7–9, resulted in significant reduction in heart weights in ErbB2^{tg} mice compared with untreated ErbB2^{tg} mice (*Figure 4D*). Together these suggest that interactions between ErbB2-βAR pathways partly underlie the hypertrophic responses observed in the ErbB2^{tg} mice vs. wild-type littermates did not show a significant reduction in heart size under β-blockers.

3.5 Acute β-adrenergic stimulation with isoproterenol increases ErbB2, β2AR, and pErk1/2 in the myocardium

Little is known about the pathways that activate and up-regulate ErbB2 in the heart. However, previous studies have shown that isoproterenol

increases ErbB2 protein in kidney[15] and salivary glands.[14] We hypothesized that βAR stimulation would have the similar effect on the myocardium. Mice injected with isoproterenol (1 mg/kg) showed significant increases in cardiac ErbB2, β2AR levels, and ERK1/2 phosphorylation at 30 min post injection (*Figure 5A*). ErbB2 levels remained elevated for 24 h (*Figure 5B*). ErbB2 mRNA (24 h) was not increased despite increased protein levels (*Figure 5C*). We hypothesized that a post-translational mechanism may be involved in ErbB2 up-regulation and we next evaluated HSP90 levels. HSP90 stabilization of ErbB2 has been previously reported[8,43] and at 30 min after isoproterenol treatment, HSP90 did in fact increase in the heart (*Figure 5A*).

3.6 Both β1- and β2-AR play an important role in ErbB2 up-regulation after β-AR activation

We then sought to determine whether β1- or β2-ARs are responsible for the up-regulation of ErbB2 and activation of downstream pathways as a consequence of isoproterenol. Mice were treated with isoproterenol (ISO) (non-specific β-agonist) alone and along with either β2-specific blocker, ICI 118 551 (*Figure 5D*) or β1-specific blocker, metoprolol (*Figure 5E*). Interestingly, ErbB2 levels increased with ICI 118 551 treatment compared with saline controls (*Figure 5D*). In contrast, there was no increase in ErbB2 levels following metoprolol alone compared with saline (*Figure 5E*). Importantly, ISO (1 mg/kg) increased ErbB2 levels significantly compared with saline, along with phosphorylation of AKT and ERK1/2. Furthermore, ISO induced up-regulation of ErbB2 despite the presence of ICI or metoprolol (*Figure 5D* and *E*). However, activation of AKT and ERK1/2 signalling pathways in response to ISO was significantly inhibited in the presence of ICI118 551 or metoprolol suggesting a complex interplay of receptors and downstream signalling pathways in ErbB2 modulation by β1- or β2-ARs.

3.7 Chronic treatment with concurrent isoproterenol and ErbB2 kinase inhibitor results in cardiac dysfunction, myocardial fibrosis, and reduced ERK1/2 and AKT phosphorylation

Since acute βAR stimulation increased ErbB2 and pERK levels, we assessed whether inhibition of ErbB2 during chronic βAR stimulation would have a negative impact on heart function, structure, and downstream signalling. Using a chronic protocol,[44,45] mice were injected with ISO (7.5 mg/kg) every 12 h for 2 weeks. Saline, DMSO, and AG825 (ErbB2 kinase inhibitor) control groups were compared with the ISO with or without AG825. Transthoracic echocardiography was performed on Day 7 and Day 14, and mice were not injected on the morning of both imaging days. Representative M-modes (*Figure 6A*) show that ISO and AG825 increased LV chamber and reduced systolic function compared with other treatments. FS (%) was not significantly different on Day 7 for all groups (not shown), but by Day 14, all of the mice in the combined ISO and AG825 treatment regimen had a significant reduction in systolic function by echocardiography (*Figure 6B*). After systolic evaluation, mice were necropsied and heart weights and tibia lengths measured. Strikingly, AG825 treatment alone significantly reduced heart weights indicating a critical role for ErbB2 in maintenance of heart size (*Figure 6C*). Representative trichrome-stained cross-sections of hearts show that the increase in blue indicative of fibrosis/collagen staining is increased in the combined treatment group (*Figure 6D* and *E*). Interestingly, fibrosis is limited to the LV free wall and the septum in all mice treated with either ISO alone or ISO along with AG825. Qualitatively, fibrosis appeared more extensive in the ISO and AG825 combined treatment group heart sections. Since hearts were sectioned for histology and molecular analysis, serial sectioning through the hearts and fibrosis quantification is a limitation in our study.

Chronic ISO treatment (*Figure 7A–C*) did not alter ErbB2 expression, but ErbB4 protein was increased, particularly the cleaved products associated with pathway activity.[38] ErbB2 kinase inhibition with AG825 markedly decreased the isoproterenol-induced ERK1/2 and AKT phosphorylation (*Figure 7D* and *E*) suggesting the key role for ErbB2 kinase in βAR-mediated activation of these downstream pathways. The protective protein bcl-XL was significantly decreased in both ISO and ISO-AG825 treatment groups (*Figure 7F*). Since β2AR and HSP90 were elevated in acute ISO treatments, we assessed levels on these proteins under chronic β-adrenergic stimulation, but neither were altered under these conditions (*Figure 7G* and *H*). Finally, increased NRG1β (neuregulin) was observed in both isoproterenol treatment groups (*Figure 7I*). Together these findings show the critical role of the active ErbB2-βAR cross-talk in cardiac remodelling.

4. Discussion

4.1 Novel mechanism of molecular and functional interaction of ErbB2 and β-adrenergic pathways in the heart

In the present study, we demonstrate that ErbB2 and βAR pathways exhibit elements of bidirectional cross-regulation. For example, ErbB2 up-regulation follows acute β-adrenergic stimulation, while ErbB2 kinase activity is protective during chronic β-adrenergic stimulation. ErbB2 kinase is involved in transactivation with an increase in pAKT and pERK after isoproterenol stimulation. Reciprocally, increasing ErbB2 expression either *in vitro* or *in vivo* increases the ratio of β2AR relative to β1AR expression. Overexpression of β2ARs increases expression of ErbB2 *in vitro*. Importantly, the induction and/or activation of ErbB2 by βAR stimulation appears to play an important feedback function, limiting the adverse effects of adrenergic activation on myocardial structure and function. Thus, the bidirectional interaction and cooperativity of these two signalling pathways appears to be critical to the long-term regulation of cardiac performance.

4.2 The role of ErbB/ NRG1β pathways in the heart

Although the cardioprotective role of the ErbB2 pathway is well known,[40,46–50] the levels of NRG1β/ErbB2/ErbB4 vary widely depending on the model used and disease stage.[9–11,51–54] Our current study shows that ErbB2 is induced with catecholamine signalling and can up-regulate the cardioprotective β2AR.[55–58] Yet, chronic ErbB2 overexpression could likely induce the hypertrophic calcineurin-NFAT signalling, which long-term may precipitate to maladaptation and heart failure.[42,59] In this context, we have observed significant up-regulation of RCAN1 (a negative regulator of calcineurin) that may account for the long-term preservation of cardiac function in ErbB2^{tg} mice. Thus, the fine-tuning control mechanisms for ErbB2 levels in the heart may represent a molecular fulcrum for balancing cardioprotection and hypertrophy.

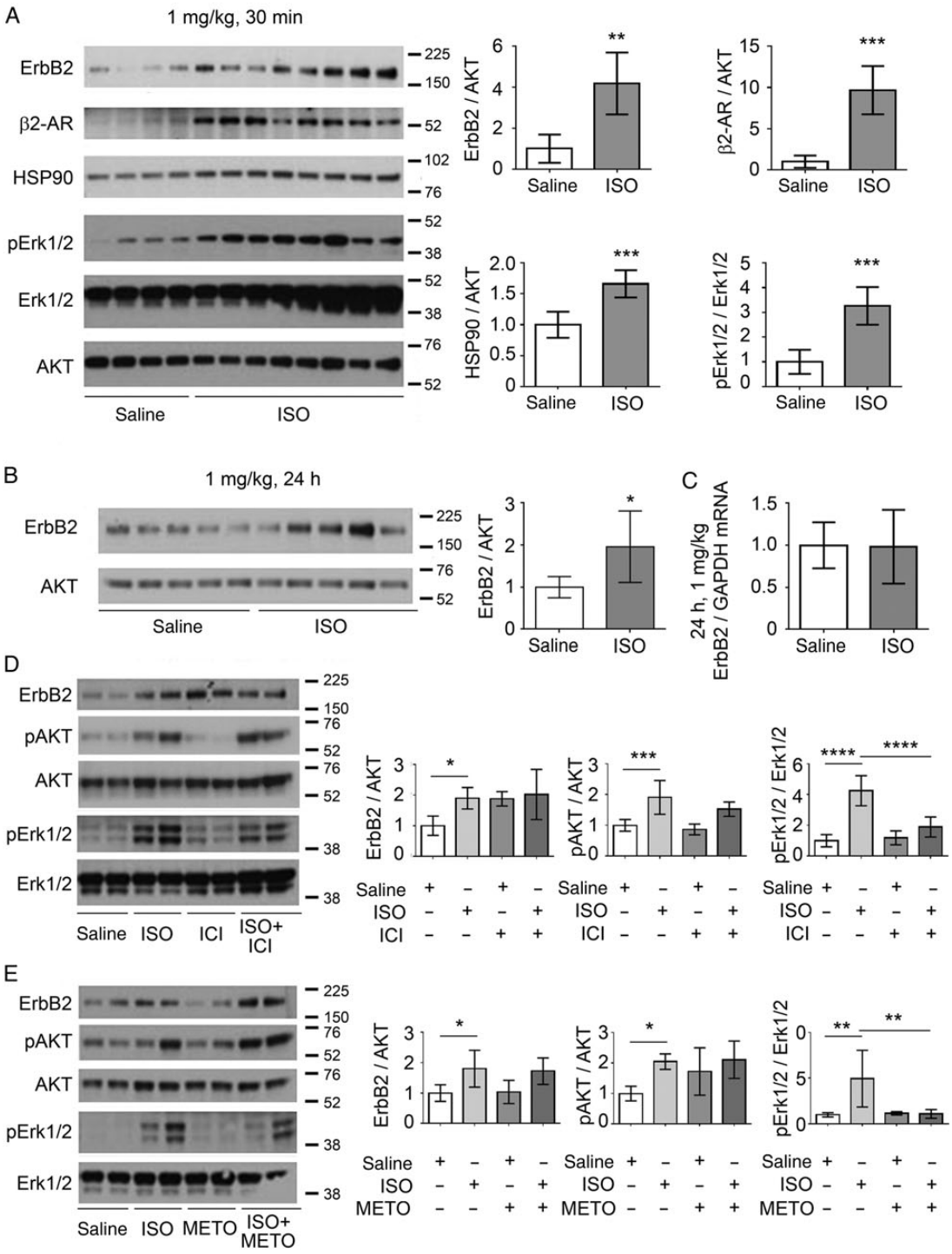

Figure 5 Acute isoproterenol stimulation effects on ErbB2 signal transduction and β2-adrenergic receptor (AR) levels. (*A*) ErbB2, β2AR, HSP90, and phospho-Erk1/2 protein levels in murine left ventricle 30 min after isoproterenol (1 mg/kg, IP) administration evaluated by western blotting and normalized by densitometry to AKT protein. (*B*) ErbB2 protein levels in murine left ventricle 24 h after isoproterenol (1 mg/kg, IP) administration evaluated by western blotting and normalized by densitometry to AKT protein. (*C*) ErbB2 mRNA levels in the left ventricle 24 h after saline or isoproterenol treatment evaluated by RT–PCR with GAPDH mRNA levels used as internal control. (*D*) ErbB2, pAKT, and pErk1/2 in murine left ventricle 30 min after isoproterenol (1 mg/kg, IP) with and without specific β2-blocker ICI 118 551 (4 mg/kg, IP) given 1 h prior to isoproterenol. (*E*) ErbB2, pAKT, and pErk1/2 levels in murine left ventricle 30 min after isoproterenol (1 mg/kg, IP) with and without specific β1-blocker metoprolol (meto) (90 mg/kg/day) in water for 5 days prior to isoproterenol. Thirty minutes after isoproterenol injection, the mice were euthanized and the hearts harvested. ErbB2 levels, Erk1/2, and AKT phosphorylation were evaluated in left ventricle lysates by western blotting, normalized to AKT protein levels and quantified by densitometry. Group size: (*A*) $n = 4$ (Saline), $n = 8$ (ISO); (*B*) $n = 5$ (Saline), $n = 5$ (ISO); (*C*) $n = 4$ (Saline), $n = 7$ (ISO), (*D*) $n = 3$ (Saline), $n = 4$ (ISO), $n = 4$ (ICI 118 551), $n = 4$ (ISO + ICI 118 551), (*E*) $n = 3$ (Saline), $n = 4$ (ISO), $n = 4$ (meto), $n = 4$ (ISO + meto). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ by Student's unpaired *t*-test or one-way ANOVA with *post hoc* Tukey's test. The data are means ± SD (fold change).

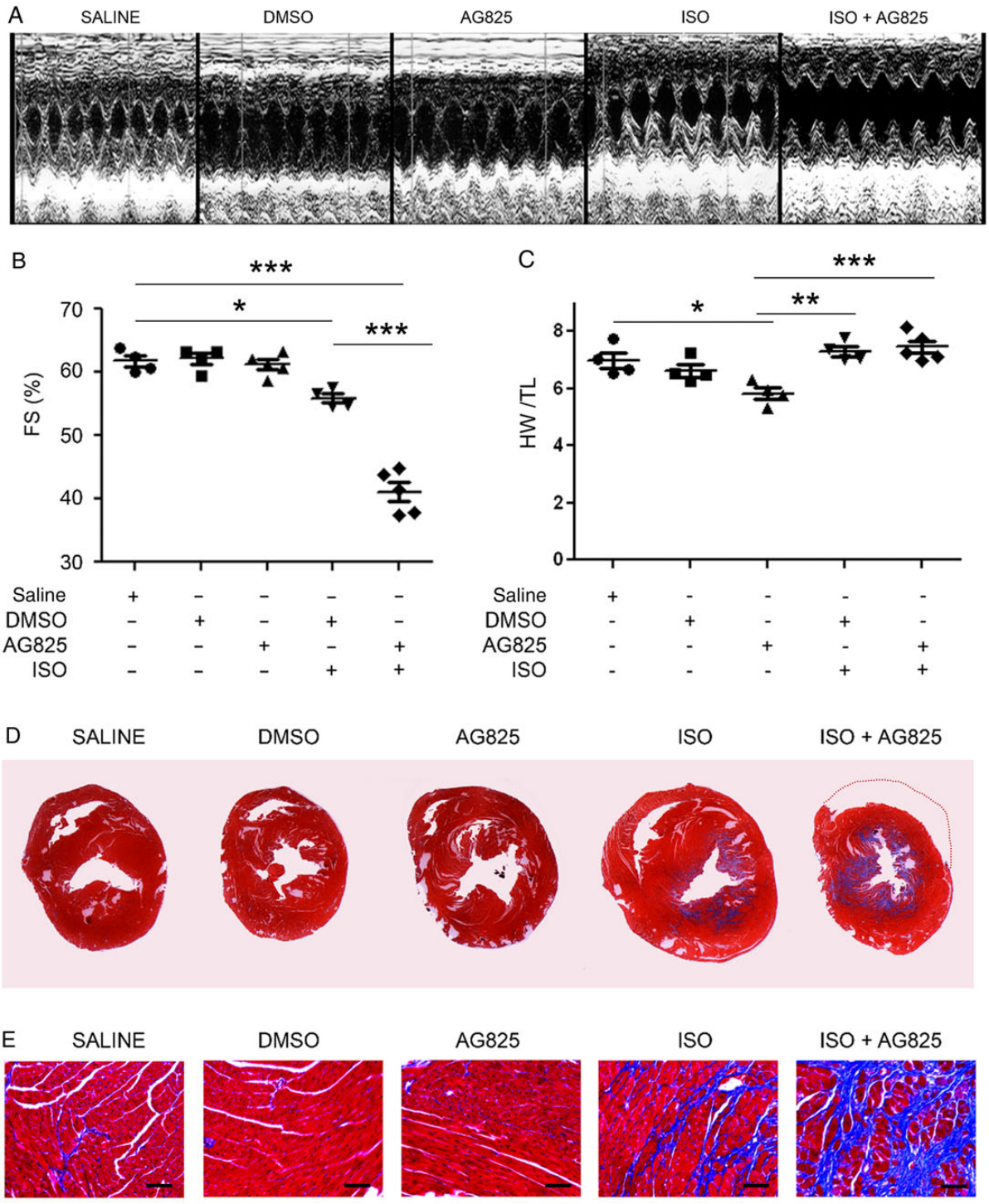

Figure 6 Chronic isoproterenol stimulation effects on cardiac morphology and function. Three- to four-month-old B6SJLF1/J female mice were divided into the following treatment groups: (1) saline, (2) DMSO (0.1%), (3) AG825 (1 mg/kg), (4) DMSO (0.1%) + ISO (7.5 mg/kg), (5) ISO (7.5 mg/kg) + AG825 (1 mg/kg), dosed both (AM and PM) for 6 days, evaluated by echocardiography on Day 7 and then treated for 6 additional days, evaluated by echocardiography on Day 14. One hour after echocardiography, a final injection (for each treatment group) was given, and mice were euthanized exactly 30 min later. All the drugs were administered subcutaneously at specific sites, ISO between shoulder blades and DMSO or AG825 in the hind left or right leg flank subcutaneous tissue. 0.1% DMSO was used as vehicle for AG825; DMSO or AG825 were given 5 min prior to ISO. Cardiac systolic function (FS, %) was evaluated by echocardiography (Day 14). Representative M-modes (*A*) and FS, % data (*B*) Day 14 are shown. (*C*) Heart weights normalized to tibia lengths. $n = 4 = 5$ (saline, DMSO, AG825, DMSO + ISO), $n = 5$ (ISO + AG825). (*D* and *E*) Cardiac fibrosis was evaluated by Masson's trichrome staining of the mid heart transverse sections from representative mice from the five treatment groups. Scale bar = 100 μm. $^{*}P < 0.05$, $^{***}P < 0.001$ by one-way ANOVA with *post hoc* Tukey's multiple comparisons test. Note: (*B*) right ventricle wall artefact (torn off) in sectioning of ISO + AG825 heart.

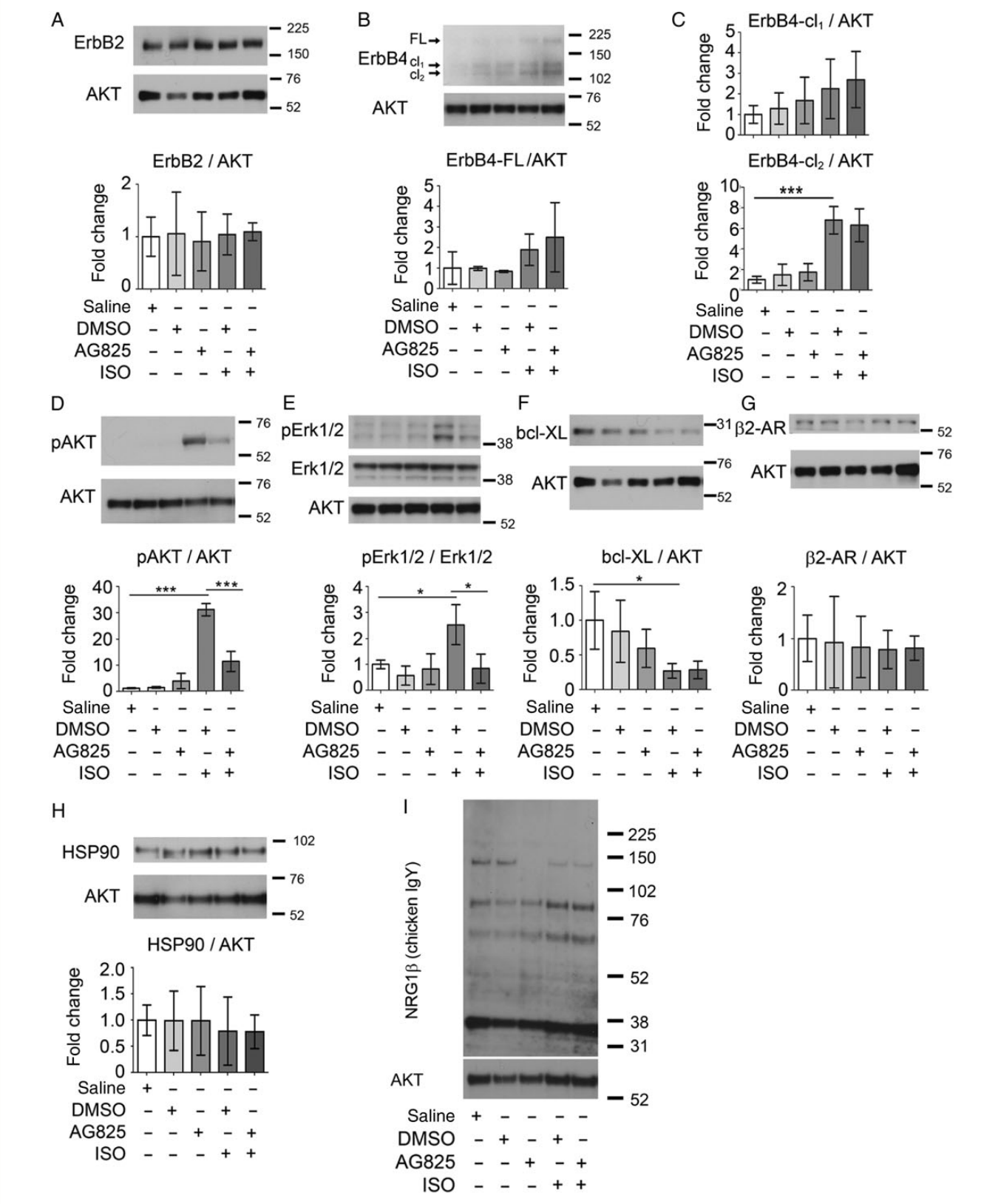

Figure 7 Chronic isoproterenol stimulation effects on ErbB signal transduction and β2-adrenergic receptor (AR). Three- to four-month-old B6SJLF1/J female mice were divided into the following treatment groups and also presented in *Figure 6*. (1) saline, (2) DMSO (0.1%), (3) AG825 (1 mg/kg), (4) DMSO (0.1%) + ISO (7.5 mg/kg), (5) ISO (7.5 mg/kg) + AG825 (1 mg/kg). The protein levels of (*A*) ErbB2, (*B* and *C*) ErbB4, (*D*) AKT phosphorylation levels, (*E*) Erk1/2 phosphorylation, (*F*) bcl-XL, (*G*) β2AR, (*H*) HSP90, (*I*) neuregulin were evaluated by western blotting, and normalized to AKT protein levels. Representative images (top) and densitometric quantifications (fold change from WT, bottom) are shown. $^{*}P < 0.05$, $^{***}P < 0.001$ by one-way ANOVA with *post hoc* Tukey's multiple comparisons test. The data are means ± SD.

Our study provides evidence that protective signalling against excessive βAR stimulation can be driven by ErbB2 due to pathway activation. One plausible mechanism for the cardioprotective effects could be through ErbB2 transactivation by ligands of GPCRs. Little is known about the role of increased ErbB2 in the heart, in contrast to the relevance of NRG1β, currently in clinical trials for heart failure.[46,47] NRG1β protective effects are thought to be due to its binding to ErbB4 (with and without ErbB2 heterodimers) and subsequent activation of protective ERK, AKT, and eNOS pathways.[36,37] In this context, the hearts of ErbB2^{tg} mice also have an increase in pAKT,[18] pERK,[18] and peNOS suggesting the involvement of ErbB2–ErbB4 heterodimers. We demonstrated in isolated cardiomyocytes that ErbB2 regulates calcium transients and SR calcium uptake, a common mechanistic feature observed with NRG1β treatment.[36,37] A comparison of our findings on ErbB2 with NRG1β literature (*Table 2*) suggests that many physiological mechanisms are shared by ErbB2 in parallel with NRG1β signalling. In that context, our studies show that ErbB2 expression in the myocytes leads to an increase of NRG1β protein levels in the hearts of ErbB2^{tg} mice. Importantly, we observed an increase in multiple cleaved forms of NRG1β using the antibodies targeting both extra- and intracellular domains.[60,61] Since NRG1β is generated by endothelial cells, our studies in the ErbB2^{tg} mice suggest the existence of a novel cross-talk between cardiomyocytes and endothelial cells, leading to a reciprocal increase of a ligand in response to the receptor overexpression.

4.3 Erbb2 induces β2AR expression and adrenergic stimulation induces ErbB2 expression

We show for the first time that ErbB2 protein up-regulates the β2AR and that β2AR expression can up-regulate ErbB2. This is further supported by the evidence showing expression of both β2AR and ErbB2 in the ErbB2^{tg} mice. Critically, lapatinib (ErbB2 and EGFR kinase inhibitor) reversed the elevation of β2AR in ErbB2^{tg} mice myocardium. This suggests that either ErbB2 or EGFR kinase is responsible for β2AR levels. Interestingly, breast cancer cells and cancer biopsies are characterized by expression of ErbB2 and β2AR.[13] On the other hand, EGFR is transactivated with βAR stimulation by ISO;[62] thus, the collaborative role of ErbB2 and EGFR in regulation of βAR signalling remains to be determined. Our studies show that ErbB2 may directly be involved in initiating β2AR up-regulation, as ErbB2 is known to maintain EGFR protein levels in cancer cells[63] and up-regulate EGFR in the hearts overexpressing ErbB2.[18]

Furthermore, our studies show that ErbB2 overexpression in the heart leads to marked increase in β2AR levels that may have implications in protective signalling. Correspondingly, studies have shown that ErbB2 and β2AR are both localized to caveolae and T-tubules in healthy cardiomyocytes.[64] Considering the reciprocal expression relationship, we identified in the current work, ErbB2 in addition to being involved in βAR levels and ratio regulation may also be playing a role in βARs localization which remains to be determined. In Schwann cells, direct adenylyl cyclase activator forskolin[65] up-regulates ErbB2 suggesting an increase in cAMP levels, which would be downstream of βAR signalling, could regulate acute levels of ErbB2. Since cAMP is increased after βARs stimulation, we investigated this connection and observed elevated cAMP and increased ErbB2 expression following β2AR expression. There are other proteins that are known to regulate levels of ErbB2.[43] In this context, we find that HSP90 to be elevated acutely by isoproterenol, and HSP90 (a heat shock protein family chaperone) is known to preserve ErbB2 protein stability and half-life.[43]

4.4 Erbb2 kinase inhibition during chronic βAR stimulation is detrimental to the heart, decreasing systolic function and reducing protective pAKT and pERK signalling

Our findings show that inhibiting ErbB2 kinase during chronic isoproterenol treatment is detrimental to heart function and structure suggesting that ErbB2 signalling is protective during chronic βAR stimulation. ErbB2 levels did not change significantly compared with control mice post chronic stimulation; however, ErbB4 and its cleaved products did increase with more cleaved receptor in the isoproterenol groups suggesting ErbB2/ErbB4/neuregulin pathway activity following βAR stimulation. ErbB2 is the preferred kinase in this receptor family and is important in the ErbB4 signalling following isoproterenol, as inhibiting ErbB2 kinase was detrimental to mice receiving both ISO and AG825. The most striking observation was the significant reduction in both pAKT and pERK following ISO and AG825 (an inhibitor of ErbB2 kinase) compared with ISO alone showing a critical *in vivo* role for ErbB2 kinase in the heart. These data indicate that ErbB2 plays a protective role potentially by transactivation mechanisms. In that context, ErbB2 transactivation by GPCR ligands has been shown in isolated cardiomyocytes[12] and in other *in vitro* assays.[66] We investigated further potential downstream factors for protection[67] and although Bcl-xL was markedly reduced in both isoproterenol groups, inhibiting ErbB2 kinase in this setting did not further decrease Bcl-xL levels. Further studies are required to understand the molecular mechanisms that drive protection when transactivation is reduced by ErbB2 kinase inhibition during chronic β-adrenergic stimulation/stress. Our study does provide novel insights into the importance of the ErbB family in cardioprotection during hypertrophic signalling due to chronic βAR stimulation.

4.5 Interactions of the ErbB2 with β-adrenergic pathways may have relevance to Trastuzumab cardiotoxicity

The proposed feedback loop mechanism (*Figure 8*) connects ErbB2 and to β-adrenergic pathways and may be relevant to cardiotoxicity observed with anti-ErbB2 therapy,[68] especially the prevention

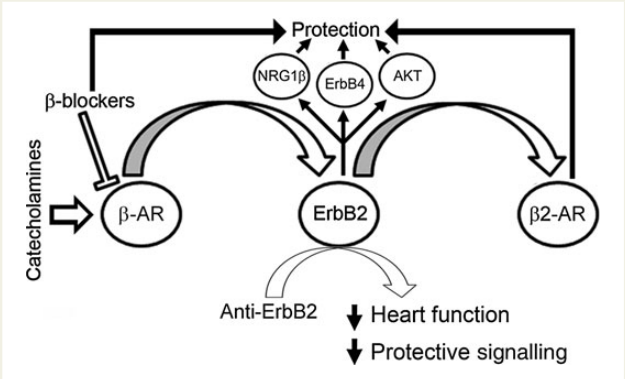

Figure 8 Schematic representation of the proposed β-adrenergic–ErbB2 signalling pathways cross-talk.

measures now in clinical trials for this cardiotoxicity. The retrospective study by Seicean *et al.*[17] found that coincidental usage of β-blockers reduced the incidence of symptomatic heart failure in breast cancer patients treated with trastuzumab, anthracyclines, or both. Since a variety of β-blockers were included in this retrospective study, pharmacological prevention with β-blockers is currently, being tested in multiple clinical trials[16,17,69] including randomized clinical trials with bisoprolol (MANTICORE 101-Breast), carvedilol (NCT01009918), and metoprolol (NCT01434134, NCT00806390) to prevent or reduce trastuzumab-induced cardiomyopathy.[16] These studies will be important to better understand how and whether specific β-blockers reduce the proposed dependency/importance of the ErbB2/ErbB4 pathway during β-adrenergic stimulation. Additionally, β-adrenergic stress is itself strongly associated with cancer progression,[70] and β-blockers also have anti-cancer effects.[71] In this context, our studies provide interesting insights into the potential cross-regulation of ErbB2 by β1- or β2-ARs. Our studies suggest in the absence of sympathetic overdrive, metoprolol reduces ErbB2 levels while in contrast, ICI118 551 increases ErbB2 levels. This observation suggests differential roles of β1AR vs. β2AR in regulating ErbB2 expression, but however ISO treatment up-regulates ErbB2 despite metoprolol. Perhaps a more rational explanation for the loss in metoprolol effects could be attributed to ISO engaging β2-ARs and thereby bypassing the inhibition mediated by β1ARs. These observations suggest that the loop between β1/β2-ARs and ErbB2 may be complex due to cross-talk of β1- and/or β2-ARs with ErbB2 being parallel and independent. This potentially indicates that using an unbiased β-blocker targeting both β1- and β2-ARs may beneficial instead of selectively targeting. Such an idea is supported by the preliminary results from the PRADA (Prevention of cardiac dysfunction during adjuvant breast cancer therapy) clinical trial presented at the 2015 American Heart Association Scientific Sessions which indicated that just using β1-blocker metoprolol may not provide sufficient protection. We believe that our studies provide the beginnings to understanding this simple yet complex cross-talk between β1/β2-ARs and ErbB2 which has significant implications in cardiomyopathy induced by the use of Herceptin.

This intriguing observation suggests that ISO treatment may activate pathways downstream of the receptors that are not blocked by β-blockers as recent studies have shown that receptors can attain different conformations and thus activate pathways that are unique. In this context, it is possible that ISO may increase HSP 90 independent of metoprolol's effects on ISO thereby increasing ErbB2 stabilization.

Here we propose that anthracyclines,[8] excessive β-adrenergic stress, or both are capable of acutely activating the ErbB2 pathway or potentially increasing cardiac ErbB2 levels sufficient to predispose to cardiac toxicity. Indeed, ErbB2 up-regulation has been clinically detected using nuclear imaging in cancer patients after anthracycline treatment[6] and only patients who showed trastuzumab cardiac binding, subsequently developed adverse cardiac outcomes.[72] Up-regulation of ErbB2 in the heart, considered a protective response, paradoxically would likely produce more trastuzumab targets inhibiting the ErbB2 pathway in the myocardium. Excessive βAR stimulation is undoubtedly common among patients, as they undergo the stressful process of cancer diagnosis and exposure to cardiotoxic anthracycline treatment. Interestingly, in agreement with our findings that ErbB2^{tg} mice hearts have increased levels of NRG1β, exposure to (anti-ErbB2) trastuzumab has been associated with reduced circulating NRG1β and an increase in blood pressure and plasma norepinephrine (NE)[73] further connecting the relationship between the ErbB2 and β-adrenergic pathways.

In summary, we have identified a novel activation loop in the heart (and *in vitro* systems) linking ErbB2 and β-adrenergic systems, where β-adrenergic receptor stimulation causes elevation and activation of ErbB2, which in turn protects the heart either by transactivation and/or by in part up-regulating β2AR, a receptor known for its protective role against excessive β-adrenergic system stimulation. Thus, the interaction and co-operation of these two signalling pathways appear to be critical to the long-term regulation of cardiac performance, with potential fallouts on pathophysiologic and therapeutic advances of cardiomyopathy induced by anti-ErbB2 antineoplastic treatments.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: none declared.

Funding

K.L.G., F.B., Y.X., J.L., X.G., B.H.K., D.B. and P.S.S. were supported by R01 HL088649 (NIH) and 15GRNT25760029 (American Heart Association, Grant-In-Aid). C.G.T. was supported by the ISHR-ES/Servier. P.P.R. and N.P. were supported by R01HL091923 (NIH) and 13GRNT17070027 (American Heart Association, Grant-In-Aid). S.V.N.P. and M.G. were supported by R01 HL 089473 (NIH). W.D.G. and X.S. were supported by 13GRNT16920028 (American Heart Association, Grant-in-Aid). D.B.S. and R.R. were supported by U01 HL100398 (NIH).

References

1. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2001;**2**:127–137.
2. Ozcelik C, Erdmann B, Pilz B, Wettschureck N, Britsch S, Hubner N, Chien KR, Birchmeier C, Garratt AN. Conditional mutation of the ErbB2 (HER2) receptor in cardiomyocytes leads to dilated cardiomyopathy. *Proc Natl Acad Sci USA* 2002;**99**:8880–8885.
3. Crone SA, Zhao YY, Fan L, Gu Y, Minamisawa S, Liu Y, Peterson KL, Chen J, Kahn R, Condorelli G, Ross J Jr, Chien KR, Lee KF. ErbB2 is essential in the prevention of dilated cardiomyopathy. *Nat Med* 2002;**8**:459–465.
4. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J, Norton L. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* 2001;**344**:783–792.
5. Tan-Chiu E, Yothers G, Romond E, Geyer CE Jr, Ewer M, Keefe D, Shannon RP, Swain SM, Brown A, Fehrenbacher L, Vogel VG, Seay TE, Rastogi P, Mamounas EP, Wolmark N, Bryant J. Assessment of cardiac dysfunction in a randomized trial comparing doxorubicin and cyclophosphamide followed by paclitaxel, with or without trastuzumab as adjuvant therapy in node-positive, human epidermal growth factor receptor 2-overexpressing breast cancer: NSABP B-31. *J Clin Oncol* 2005;**23**:7811–7819.
6. de Korte MA, de Vries EG, Lub-de Hooge MN, Jager PL, Gietema JA, van der Graaf WT, Sluiter WJ, van Veldhuisen DJ, Suter TM, Sleijfer DT, Perik PJ. 111Indium-trastuzumab visualises myocardial human epidermal growth factor receptor 2 expression shortly after anthracycline treatment but not during heart failure: a clue to uncover the mechanisms of trastuzumab-related cardiotoxicity. *Eur J Cancer* 2007;**43**:2046–2051.
7. Ewer MS, Ewer SM. Troponin I provides insight into cardiotoxicity and the anthracycline-trastuzumab interaction. *J Clin Oncol* 2010;**28**:3901–3904.
8. Gabrielson K, Bedja D, Pin S, Tsao A, Gama L, Yuan B, Muratore N. Heat shock protein 90 and ErbB2 in the cardiac response to doxorubicin injury. *Cancer Res* 2007;**67**:1436–1441.
9. Doggen K, Ray L, Mathieu M, Mc Entee K, Lemmens K, De Keulenaer GW. Ventricular ErbB2/ErbB4 activation and downstream signaling in pacing-induced heart failure. *J Mol Cell Cardiol* 2009;**46**:33–38.
10. Lemmens K, Segers VF, Demolder M, De Keulenaer GW. Role of neuregulin-1/ErbB2 signaling in endothelium-cardiomyocyte cross-talk. *J Biol Chem* 2006;**281**:19469–19477.
11. Naga Prasad SV, Duan ZH, Gupta MK, Surampudi VS, Volinia S, Calin GA, Liu CG, Kotwal A, Moravec CS, Starling RC, Perez DM, Sen S, Wu Q, Plow EF, Croce CM, Karnik S. Unique microRNA profile in end-stage heart failure indicates alterations in specific cardiovascular signaling networks. *J Biol Chem* 2009;**284**:27487–27499.
12. Negro A, Brar BK, Gu Y, Peterson KL, Vale W, Lee KF. erbB2 is required for G protein-coupled receptor signaling in the heart. *Proc Natl Acad Sci USA* 2006;**103**:15889–15893.

13. Shi M, Liu D, Duan H, Qian L, Wang L, Niu L, Zhang H, Yong Z, Gong Z, Song L, Yu M, Hu M, Xia Q, Shen B, Guo N. The beta2-adrenergic receptor and Her2 comprise a positive feedback loop in human breast cancer cells. *Breast Cancer Res Treat* 2011; **125**:351–362.
14. Kelentey B, Kerr M, Tao Z, Purushotham KR, Humphreys-Beher MG, Zelles T. Inhibition of rat parotid gland growth response induced by chronic isoproterenol following treatment with quinolone antibiotics. *Mol Cell Biochem* 1996;**165**:55–63.
15. Camprecios G, Sanchez-Vizcaino E, Soley M, Ramirez I. Chronic beta-adrenergic stimulation increases ErbB receptors and cell proliferation in mouse kidney. *Growth Factors* 2011;**29**:94–101.
16. Nohria A. Beta-adrenergic blockade for anthracycline- and trastuzumab-induced cardiotoxicity: is prevention better than cure? *Circ Heart Fail* 2013;**6**:358–361.
17. Seicean S, Seicean A, Alan N, Plana JC, Budd GT, Marwick TH. Cardioprotective effect of beta-adrenoceptor blockade in patients with breast cancer undergoing chemotherapy: follow-up study of heart failure. *Circ Heart Fail* 2013;**6**:420–426.
18. Sysa-Shah P, Xu Y, Guo X, Belmonte F, Kang B, Bedja D, Pin S, Tsuchiya N, Gabrielson K. Cardiac-specific over-expression of epidermal growth factor receptor 2 (ErbB2) induces pro-survival pathways and hypertrophic cardiomyopathy in mice. *PLoS One* 2012;**7**:e42805.
19. Zhang W, Yano N, Deng M, Mao Q, Shaw SK, Tseng YT. Beta-adrenergic receptor-PI3 K signaling crosstalk in mouse heart: elucidation of immediate downstream signaling cascades. *PLoS ONE* 2011;**6**:e26581.
20. Becher PM, Lindner D, Miteva K, Savvatis K, Zietsch C, Schmack B, Van Linthout S, Westermann D, Schultheiss HP, Tschope C. Role of heart rate reduction in the prevention of experimental heart failure: comparison between If-channel blockade and beta-receptor blockade. *Hypertension* 2012;**59**:949–957.
21. Murray DR, Mummidi S, Valente AJ, Yoshida T, Somanna NK, Delafontaine P, Dinarello CA, Chandrasekar B. Beta2 adrenergic activation induces the expression of IL-18 binding protein, a potent inhibitor of isoproterenol induced cardiomyocyte hypertrophy in vitro and myocardial hypertrophy in vivo. *J Mol Cell Cardiol* 2012;**52**: 206–218.
22. Habashi JP, Judge DP, Holm TM, Cohn RD, Loeys BL, Cooper TK, Myers L, Klein EC, Liu G, Calvi C, Podowski M, Neptune ER, Halushka MK, Bedja D, Gabrielson K, Rifkin DB, Carta L, Ramirez F, Huso DL, Dietz HC. Losartan, an AT1 antagonist, prevents aortic aneurysm in a mouse model of Marfan syndrome. *Science* 2006;**312**: 117–121.
23. Xiao RP, Zhang SJ, Chakir K, Avdonin P, Zhu W, Bond RA, Balke CW, Lakatta EG, Cheng H. Enhanced G(i) signaling selectively negates beta2-adrenergic receptor (AR)--but not beta1-AR-mediated positive inotropic effect in myocytes from failing rat hearts. *Circulation* 2003;**108**:1633–1639.
24. Tocchetti CG, Wang W, Froehlich JP, Huke S, Aon MA, Wilson GM, Di Benedetto G, O'Rourke B, Gao WD, Wink DA, Toscano JP, Zaccolo M, Bers DM, Valdivia HH, Cheng H, Kass DA, Paolocci N. Nitroxyl improves cellular heart function by directly enhancing cardiac sarcoplasmic reticulum Ca2+ cycling. *Circ Res* 2007;**100**:96–104.
25. Gao WD, Perez NG, Marban E. Calcium cycling and contractile activation in intact mouse cardiac muscle. *J Physiol* 1998;**507**(Pt 1):175–184.
26. Perrino C, Naga Prasad SV, Schroder JN, Hata JA, Milano C, Rockman HA. Restoration of beta-adrenergic receptor signaling and contractile function in heart failure by disruption of the betaARK1/phosphoinositide 3-kinase complex. *Circulation* 2005;**111**: 2579–2587.
27. Naga Prasad SV, Barak LS, Rapacciuolo A, Caron MG, Rockman HA. Agonist-dependent recruitment of phosphoinositide 3-kinase to the membrane by beta-adrenergic receptor kinase 1. A role in receptor sequestration. *J Biol Chem* 2001;**276**:18953–18959.
28. Kilts JD, Akazawa T, Richardson MD, Kwatra MM. Age increases cardiac Galpha(i2) expression, resulting in enhanced coupling to G protein-coupled receptors. *J Biol Chem* 2002;**277**:31257–31262.
29. Vasudevan NT, Mohan ML, Gupta MK, Hussain AK, Naga Prasad SV. Inhibition of protein phosphatase 2A activity by PI3Kgamma regulates beta-adrenergic receptor function. *Mol Cell* 2011;**41**:636–648.
30. Koch L, Wunderlich FT, Seibler J, Konner AC, Hampel B, Irlenbusch S, Brabant G, Kahn CR, Schwenk F, Bruning JC. Central insulin action regulates peripheral glucose and fat metabolism in mice. *J Clin Invest* 2008;**118**:2132–2147.
31. Goruppi S, Patten RD, Force T, Kyriakis JM. Helix-loop-helix protein p8, a transcriptional regulator required for cardiomyocyte hypertrophy and cardiac fibroblast matrix metalloprotease induction. *Mol Cell Biol* 2007;**27**:993–1006.
32. Leo C, Sala V, Morello M, Chiribiri A, Riess I, Mancardi D, Schiaffino S, Ponzetto C, Crepaldi T. Activated Met signalling in the developing mouse heart leads to cardiac disease. *PLoS ONE* 2011;**6**:e14675.
33. Sahn DJ, DeMaria A, Kisslo J, Weyman A. Recommendations regarding quantitation in M-mode echocardiography: results of a survey of echocardiographic measurements. *Circulation* 1978;**58**:1072–1083.
34. Head BP, Patel HH, Roth DM, Lai NC, Niesman IR, Farquhar MG, Insel PA. G-protein-coupled receptor signaling components localize in both sarcolemmal and intracellular caveolin-3-associated microdomains in adult cardiac myocytes. *J Biol Chem* 2005;**280**:31036–31044.
35. Nagy P, Vereb G, Sebestyen Z, Horvath G, Lockett SJ, Damjanovich S, Park JW, Jovin TM, Szollosi J. Lipid rafts and the local density of ErbB proteins influence the biological role of homo- and heteroassociations of ErbB2. *J Cell Sci* 2002;**115**:4251–4262.
36. Brero A, Ramella R, Fitou A, Dati C, Alloatti G, Gallo MP, Levi R. Neuregulin-1beta1 rapidly modulates nitric oxide synthesis and calcium handling in rat cardiomyocytes. *Cardiovasc Res* 2010;**88**:443–452.
37. Lemmens K, Fransen P, Sys SU, Brutsaert DL, De Keulenaer GW. Neuregulin-1 induces a negative inotropic effect in cardiac muscle: role of nitric oxide synthase. *Circulation* 2004;**109**:324–326.
38. Yamamoto H, Higa-Nakamine S, Noguchi N, Maeda N, Kondo Y, Toku S, Kukita I, Sugahara K. Desensitization by different strategies of epidermal growth factor receptor and ErbB4. *J Pharmacol Sci* 2014;**124**:287–293.
39. Chang AN, Huang J, Battiprolu PK, Hill JA, Kamm KE, Stull JT. The effects of neuregulin on cardiac Myosin light chain kinase gene-ablated hearts. *PLoS ONE* 2013;**8**:e66720.
40. Timolati F, Ott D, Pentassuglia L, Giraud MN, Perriard JC, Suter TM, Zuppinger C. Neuregulin-1 beta attenuates doxorubicin-induced alterations of excitation-contraction coupling and reduces oxidative stress in adult rat cardiomyocytes. *J Mol Cell Cardiol* 2006;**41**:845–854.
41. Vega RB, Rothermel BA, Weinheimer CJ, Kovacs A, Naseem RH, Bassel-Duby R, Williams RS, Olson EN. Dual roles of modulatory calcineurin-interacting protein 1 in cardiac hypertrophy. *Proc Natl Acad Sci USA* 2003;**100**:669–674.
42. Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J, Grant SR, Olson EN. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell* 1998;**93**:215–228.
43. Peng X, Guo X, Borkan SC, Bharti A, Kuramochi Y, Calderwood S, Sawyer DB. Heat shock protein 90 stabilization of ErbB2 expression is disrupted by ATP depletion in myocytes. *J Biol Chem* 2005;**280**:13148–13152.
44. Oudit GY, Crackower MA, Eriksson U, Sarao R, Kozieradzki I, Sasaki T, Irie-Sasaki J, Gidrewicz D, Rybin VO, Wada T, Steinberg SF, Backx PH, Penninger JM. Phosphoinositide 3-kinase gamma-deficient mice are protected from isoproterenol-induced heart failure. *Circulation* 2003;**108**:2147–2152.
45. Camprecios G, Navarro M, Soley M, Ramirez I. Acute and chronic adrenergic stimulation of submandibular salivary glands. Effects on the endocrine function of epidermal growth factor in mice. *Growth Factors* 2009;**27**:300–308.
46. Gao R, Zhang J, Cheng L, Wu X, Dong W, Yang X, Li T, Liu X, Xu Y, Li X, Zhou M. A Phase II, randomized, double-blind, multicenter, based on standard therapy, placebo-controlled study of the efficacy and safety of recombinant human neuregulin-1 in patients with chronic heart failure. *J Am Coll Cardiol* 2010;**55**:1907–1914.
47. Jabbour A, Hayward CS, Keogh AM, Kotlyar E, McCrohon JA, England JF, Amor R, Liu X, Li XY, Zhou MD, Graham RM, Macdonald PS. Parenteral administration of recombinant human neuregulin-1 to patients with stable chronic heart failure produces favourable acute and chronic haemodynamic responses. *Eur J Heart Fail* 2011;**13**: 83–92.
48. Liu FF, Stone JR, Schuldt AJ, Okoshi K, Okoshi MP, Nakayama M, Ho KK, Manning WJ, Marchionni MA, Lorell BH, Morgan JP, Yan X. Heterozygous knockout of neuregulin-1 gene in mice exacerbates doxorubicin-induced heart failure. *Am J Physiol Heart Circ Physiol* 2005;**289**:H660–e42666.
49. Liu X, Gu X, Li Z, Li X, Li H, Chang J, Chen P, Jin J, Xi B, Chen D, Lai D, Graham RM, Zhou M. Neuregulin-1/erbB-activation improves cardiac function and survival in models of ischemic, dilated, and viral cardiomyopathy. *J Am Coll Cardiol* 2006;**48**:1438–1447.
50. Sawyer DB, Zuppinger C, Miller TA, Eppenberger HM, Suter TM. Modulation of anthracycline-induced myofibrillar disarray in rat ventricular myocytes by neuregulin-1 beta and anti-erbB2: potential mechanism for trastuzumab-induced cardiotoxicity. *Circulation* 2002;**105**:1551–1554.
51. Rohrbach S, Niemann B, Silber RE, Holtz J. Neuregulin receptors erbB2 and erbB4 in failing human myocardium—depressed expression and attenuated activation. *Basic Res Cardiol* 2005;**100**:240–249.
52. Rohrbach S, Yan X, Weinberg EO, Hasan F, Bartunek J, Marchionni MA, Lorell BH. Neuregulin in cardiac hypertrophy in rats with aortic stenosis. Differential expression of erbB2 and erbB4 receptors. *Circulation* 1999;**100**:407–412.
53. Uray IP, Connelly JH, Thomazy V, Shipley GL, Vaughn WK, Frazier OH, Taegtmeyer H, Davies PJ. Left ventricular unloading alters receptor tyrosine kinase expression in the failing human heart. *J Heart Lung Transplant* 2002;**21**:771–782.
54. De Keulenaer GW, Doggen K, Lemmens K. The vulnerability of the heart as a pluricellular paracrine organ: lessons from unexpected triggers of heart failure in targeted ErbB2 anticancer therapy. *Circ Res* 2010;**106**:35–46.
55. Milano CA, Allen LF, Rockman HA, Dolber PC, McMinn TR, Chien KR, Johnson TD, Bond RA, Lefkowitz RJ. Enhanced myocardial function in transgenic mice overexpressing the beta 2-adrenergic receptor. *Science* 1994;**264**:582–586.
56. Patterson AJ, Zhu W, Chow A, Agrawal R, Kosek J, Xiao RP, Kobilka B. Protecting the myocardium: a role for the beta2 adrenergic receptor in the heart. *Crit Care Med* 2004; **32**:1041–1048.
57. Chruscinski AJ, Rohrer DK, Schauble E, Desai KH, Bernstein D, Kobilka BK. Targeted disruption of the beta2 adrenergic receptor gene. *J Biol Chem* 1999;**274**:16694–16700.
58. Zheng M, Zhu W, Han Q, Xiao RP. Emerging concepts and therapeutic implications of beta-adrenergic receptor subtype signaling. *Pharmacol Ther* 2005;**108**:257–268.
59. Maillet M, van Berlo JH, Molkentin JD. Molecular basis of physiological heart growth: fundamental concepts and new players. *Nat Rev Mol Cell Biol* 2013;**14**:38–48.

60. Cote GM, Miller TA, Lebrasseur NK, Kuramochi Y, Sawyer DB. Neuregulin-1alpha and beta isoform expression in cardiac microvascular endothelial cells and function in cardiac myocytes in vitro. *Exp Cell Res* 2005;**311**:135–146.
61. Kuramochi Y, Cote GM, Guo X, Lebrasseur NK, Cui L, Liao R, Sawyer DB. Cardiac endothelial cells regulate reactive oxygen species-induced cardiomyocyte apoptosis through neuregulin-1beta/erbB4 signaling. *J Biol Chem* 2004;**279**:51141–51147.
62. Grisanti LA, Talarico JA, Carter RL, Yu JE, Repas AA, Radcliffe SW, Tang HA, Makarewich CA, Houser SR, Tilley DG. Beta-adrenergic receptor-mediated transactivation of epidermal growth factor receptor decreases cardiomyocyte apoptosis through differential subcellular activation of ERK1/2 and Akt. *J Mol Cell Cardiol* 2014;**72**:39–51.
63. Grassian AR, Schafer ZT, Brugge JS. ErbB2 stabilizes epidermal growth factor receptor (EGFR) expression via Erk and Sprouty2 in extracellular matrix-detached cells. *J Biol Chem* 2011;**286**:79–90.
64. Ueda H, Oikawa A, Nakamura A, Terasawa F, Kawagishi K, Moriizumi T. Neuregulin receptor ErbB2 localization at T-tubule in cardiac and skeletal muscle. *J Histochem Cytochem* 2005;**53**:87–91.
65. Fregien NL, White LA, Bunge MB, Wood PM. Forskolin increases neuregulin receptors in human Schwann cells without increasing receptor mRNA. *Glia* 2005;**49**:24–35.
66. Daub H, Weiss FU, Wallasch C, Ullrich A. Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. *Nature* 1996;**379**:557–560.
67. Grazette LP, Boecker W, Matsui T, Semigran M, Force TL, Hajjar RJ, Rosenzweig A. Inhibition of ErbB2 causes mitochondrial dysfunction in cardiomyocytes: implications for herceptin-induced cardiomyopathy. *J Am Coll Cardiol* 2004;**44**:2231–2238.
68. Yancy CW, Jessup M, Bozkurt B, Butler J, Casey DE Jr., Drazner MH, Fonarow GC, Geraci SA, Horwich T, Januzzi JL, Johnson MR, Kasper EK, Levy WC, Masoudi FA, McBride PE, McMurray JJ, Mitchell JE, Peterson PN, Riegel B, Sam F, Stevenson LW, Tang WH, Tsai EJ, Wilkoff BL. 2013 ACCF/AHA guideline for the management of heart failure: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. *Circulation* 2013;**128**: e240–e319.
69. Kalam K, Marwick TH. Role of cardioprotective therapy for prevention of cardiotoxicity with chemotherapy: a systematic review and meta-analysis. *Eur J Cancer* 2013;**49**: 2900–2909.
70. Antoni MH, Lutgendorf SK, Cole SW, Dhabhar FS, Sephton SE, McDonald PG, Stefanek M, Sood AK. The influence of bio-behavioural factors on tumour biology: pathways and mechanisms. *Nat Rev Cancer* 2006;**6**:240–248.
71. Melhem-Bertrandt A, Chavez-Macgregor M, Lei X, Brown EN, Lee RT, Meric-Bernstam F, Sood AK, Conzen SD, Hortobagyi GN, Gonzalez-Angulo AM. Beta-blocker use is associated with improved relapse-free survival in patients with triple-negative breast cancer. *J Clin Oncol* 2011;**29**:2645–2652.
72. Behr TM, Behe M, Wormann B. Trastuzumab and breast cancer. *N Engl J Med* 2001;**345**: 995–996.
73. Lenneman CG, Abdallah WM, Smith HM, Abramson V, Mayer IA, Silverstein C, Silverstein C, Means-Powell J, Paranjape SY, Lenihan D, Sawyer DB, Raj SR. Sympathetic nervous system alterations with HER2+ antagonism: an early marker of cardiac dysfunction with breast cancer treatment? *Ecancermedicalscience* 2014;**8**:446.

Corrigendum

doi:10.1093/cvr/cvv269
Online publish-ahead-of-print 30 December 2015

Corrigendum to: miR-30e targets IGF2-regulated osteogenesis in bone marrow-derived mesenchymal stem cells, aortic smooth muscle cells, and ApoE$^{-/-}$ mice [Cardiovasc Res 2015; 106(1):131–142]

There were some references to the reported concentration of a standard reagent used which were incorrect. This does not affect the conclusion of the paper in any way but the author would like to amend this. It is shown correctly below and has also been corrected online. The authors apologise for this error.

In Figure 4 the legend, 500 mg/ml should be substituted with 500 ng/ml.

In section 3.8 IGF2 recombinant protein rescues miR-30e-repressed osteogenesis in SMCs there are two unit errors; one is 500 mg/ml should be corrected to 500 ng/ml; the second is 250 mg/ml should be corrected to 250 ng/ml.