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# Stimulation of the four isoforms of receptor tyrosine kinase ErbB4, but not ErbB1, confers cardiomyocyte hypertrophy

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## Abstract

Epidermal growth factor (EGF) receptors (ErbB1-ErbB4) promote cardiac development and growth, although the specific EGF ligands and receptor isoforms involved in growth/repair versus pathology remain undefined. We challenged ventricular cardiomyocytes with EGF-like ligands and observed that selective activation of ErbB4 (the receptor for neuregulin 1, NRG1), but not ErbB1 (the receptor for EGF, EGFR), stimulated hypertrophy. This lack of direct ErbB1-mediated hypertrophy occurred despite robust activation of extracellular-regulated kinase 1/2 (ERK) and Akt. Hypertrophic responses to NRG1 were unaffected by the tyrosine kinase inhibitor (AG1478) at concentrations that are selective for ErbB1 over ErbB4. NRG1-induced cardiomyocyte enlargement was suppressed by siRNA knockdown of ErbB4 and ErbB2, whereas ERK phosphorylation was only suppressed by ErbB4 siRNA. Four ErbB4 isoforms exist (JM-a/JM-b and CYT-1/CYT-2), generated by alternative splicing, and their expression declines postnatally and following cardiac hypertrophy. Silencing of all four isoforms in cardiomyocytes, using an ErbB4 siRNA, abrogated NRG1-induced hypertrophic promoter/reporter activity, which was rescued by co-expression of knockdown-resistant versions of the ErbB4 isoforms. Thus, ErbB4 confers cardiomyocyte hypertrophy to NRG1, and all four ErbB4 isoforms possess the capacity to mediate this effect.


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## Introduction

Pathological cardiac hypertrophy is an independent risk factor for heart failure and sudden death, and is characterized by the enlargement of individual cardiomyocytes, remodeling of the actin cytoskeleton, and upregulation of hypertrophic genes (Frey and Olson, 2003; Marian and Braunwald, 2017). Cardiomyocyte hypertrophy can be induced by local factors, such as neuregulin 1 (NRG1, a member of the epidermal growth factor ligand family) (Zhao et al., 1998), as well as a variety of physiological (e.g. pregnancy or exercise training) and pathological stimuli including hypertension and the vasoactive peptide, angiotensin II (AngII) (Brandt et al., 2012; Eghbali et al., 2005; Pelliccia et al., 1991; Sadoshima and Izumo, 1993). Apart from NRG1, 14 other EGF-like ligands exist (Wilson et al., 2009), although their contribution to cardiac function is less clear.

The epidermal growth factor receptors (ErbBs) are a family of tyrosine kinase receptors that regulate cell growth, survival and differentiation (Burgess, 2008). Upon activation by EGF ligands, ErbB receptors form hetero- or homo-dimers and phosphorylate intracellular tyrosine residues to activate downstream signaling pathways. They have four subtypes (ErbB1 - 4) (Burgess, 2008). ErbB1 (also known as the Epidermal Growth Factor Receptor, EGFR), ErbB2 and ErbB4 are abundant in postnatal cardiomyocytes and are essential for cardiac development and normal function in the adult heart (D'Uva et al., 2015; Gassmann et al., 1995; Lee et al., 1995; Reichelt et al., 2017; Threadgill et al., 1995; Vermeulen et al., 2016; Wadugu and Kuhn, 2012), ErbB2, which cannot bind ligands, is the preferred dimerization partner for ErbB1 and ErbB4 (Burgess, 2008).

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The ErbB4 selective agonist NRG1 is produced by vascular endothelial cells and activates ErbB4 on cardiomyocytes in a paracrine manner to regulate cardiac physiology (Parodi and Kuhn, 2014). *In vitro*, NRG1 induces robust cardiomyocyte hypertrophy, presumably via ErbB4 (Baliga et al., 1999; Zhao et al., 1998). ErbB4 has four main isoforms due to alternative splicing: JM-a CYT-1, JM-a CYT-2, JM-b CYT-1 and JM-b CYT-2 (Veikkolainen et al., 2011). These four isoforms are structurally different in two domains, the juxtamembrane (JM) domain and the cytoplasmic (CYT) domain; The JM-a, but not JM-b, isoform allows shedding of the ectodomain by metalloproteases (Veikkolainen et al., 2011), whereas the CYT-1 isoform has an additional 16 amino acids, which allows binding with PI3K (phosphoinositide 3-kinase) (Veikkolainen et al., 2011) and ubiquitin ligases. (Sundvall et al., 2008) The four isoforms of ErbB4 have profound differences in their function in many tissues; for example, the JM-a isoforms are capable of suppressing astrocyte differentiation, whereas the JM-b isoforms are not (Sardi et al., 2006). The function of different isoforms in cardiomyocytes remains uninvestigated.

In this study, we evaluated the influence of growth receptor ligands on cardiomyocyte hypertrophy, and used loss- and gain-of-function strategies to investigate the role of the four ErbB isoforms in mediating the hypertrophic response.

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## Materials and methods

*Animals*

All experiments were conducted in accordance with the Australian Code of Practice for Care and Use of Animals for Scientific Purposes and approved by the institutional ethics committees of the University of Queensland. RNA was isolated from CD-1 mouse hearts at embryonic days 10.5, 13.5, 16.5, and 18.5, and postnatal days 1, 6, 14 and 28. RNA was also obtained from adult mice with cardiac-specific overexpression of the calcium-dependent phosphatase calcineurin. These mice develop cardiac hypertrophy soon after birth and reached maximal hypertrophy at 8 weeks of age, progressing to dilated cardiomyopathy and heart failure (Molkentin et al., 1998).

*Myocyte culture*

Neonatal rat ventricular myocytes (NRVM) were isolated from ventricles of 1-2 day old Sprague Dawley rats and purified via gradient centrifugation as previously described (Thomas et al., 2002). To mimic the up-regulation of $AT_1R$ during hypertrophy, $AT_1R$ was delivered into cardiomyocytes with DNA transfection or adenovirus (Thomas et al., 2002) as required and indicated in the figures. Cardiomyocytes were stimulated with the following hypertrophic agents at concentrations as indicated in graphs: AngII, NRG1 (NRG1-β1), EGF, amphiregulin, betacellulin, transforming growth factor α, epiregulin, epigen, HB-EGF, neuregulins -1α, -1β1, -2α, -2β, -3 and -4. To induce the activation of ERK, promoter activity of hypertrophic genes and hypertrophic growth (increase of protein to DNA ratio), cardiomyocytes were treated with indicated agonists for 5min, 48h and 48-72h separately. The ErbB receptor antagonist, AG1478, was added to cells 30 min



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before stimulation at concentrations, as indicated.

*Hypertrophy assay*

Cells were lysed in TE buffer containing 0.06% SDS for 4h at room temperature, and assayed for both protein (BCA assay, Thermo Scientific) and DNA content (Picogreen dsDNA assay kit, Life Technologies). Before the DNA assay, cell lysates were treated with 0.2g/L proteinase K (Life Technologies) overnight. Hypertrophy was defined by the increase in the total protein in the absence of a change in DNA levels (Thomas et al., 2002).

*Phalloidin stain*

Cardiomyocytes were fixed with 4% paraformaldehyde followed by staining with Alexa594-labelled phalloidin as per the manufacturer's protocol (Invitrogen) and imaged on a confocal microscope (400x magnification, Olympus FV1000 Inverted Confocal, Japan) to reflect the F-actin reorganization into the sarcomere.

*siRNA transfection*

The day after cardiomyocyte isolation, siRNAs specific for ErbB2 and ErbB4 were mixed with Lipofectamine® RNAiMAX (Life Technologies) and treansfected into cells at a final concentration of 20nM. Non-targeting siRNA was used as a control. ErbB2 (L-090224-02), ErbB4 (L-080170-02) and non-targeting siRNA (D-001810-10) was predesigned and purchased from Dharmacon (ON-TARGETplusSMARTpool). Cardiomyocytes were transfected with siRNA 48h before treatment with agonists.

*Plasmid constructs*

The shRNA for ErbB2 and ErbB4 were designed using online BLOCK-iT™ RNAi Designer program (Invitrogen). To achieve high knockdown rates, the shRNA was



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engineering into a microRNA 155 backbone by introducing a stem-loop structure. DNA oligonucleotides corresponding to each shRNA were synthesised and cloned into BLOCK-iT™ Pol II miR RNAi Expression Vector (Invitrogen) as per manufacturer's protocols.

*Generation of knockdown-resistant ErbB4 constructs*

To generate ErbB4 expression constructs that are resistant to knockdown by the ErbB4 shRNA, a segment common to all four ErbB4 isoforms (residues 1-1455) encompassing the shRNA targeting site, was excised from the pIRESpuro2 vector containing the ErbB4 coding region and subcloned into the pSL301 vector via NheI and HpaI digestion. PCR mutagenesis was performed on the pSL301/ErbB4 (1-1455) plasmid using the kdrErbB4 primers designed to introduce mutations in the shRNA target site of ErbB4 (i.e., the kdrErbB4 primer forward 5'-CTACATCACGGACAACTCCAATCTCTGCTACTACCACACCATCAAC; reverse 5'-GTTGATGGTGTGGTAGTAGCAGAGATTGGAGTTGTCCGTGATGTAG). The PCR products were transformed into XL1-blue competent cells and the introduction of silent mutations was confirmed by sequencing. The common, mutated segment (1-1455) was subcloned back into the four individual ErbB4 constructs in pIRESpuro2 via NheI and HpaI digestion and ligation. Positive clones were determined by sequencing.

*DNA transfection and luciferase reporter assay*

Reporter assays were used to measure activation of the prototypic hypertrophic genes. Luciferase reporter plasmids driven by hypertrophic gene promoter (MLC-2V, ANP-328, and cyclin D) were gifts from Dr Mona Nemer (University of Ottawa,



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Canada). Cardiomyocytes, seeded in 12-well plates, were transiently transfected with 300ng plasmid DNA for the luciferase reporter constructs, along with other DNA constructs (100ng $AT_1R$ (Figure 1) and 100ng shRNA targeting specific ErbBs, and 100ng knockdown resistant ErbB4 isoforms (Figure 6) or 20-100ng knockdown resistant ErbB4 isoforms (Figure 6)) using Lipofectamine™ 2000 (Life Technologies). The hypertrophic signaling was induced by treatment with AngII, NRG1 or EGF for 48h. The cells were harvested and assayed with a luciferase assay kit (Promega).

*Western blot*

Western blots of cellular protein lysates were probed with primary antibodies to ErbB4 (sc-283, Santa Cruz), ErbB2 (Ab-3, Calbiochem), p-ERK (#9106, Cell Signalling), ERK(#4695, Cell Signalling) and β-actin (AC-15, Sigma), phospho-Akt-Ser473 (#9271, Cell Signalling), Akt (#9272, Cell Signalling). IRDye-conjugated secondary antibodies were used before imaging with LI-COR Odyssey infrared imaging system (LI-COR biotechnology). LI-COR Image Studio Lite software (version 3.1) was used to quantify protein expression.

*Absolute quantification real-time PCR*

RNA was extracted from animal tissues or cultured cells using TRIzol reagent (Invitrogen) and cDNA was synthesized with the SuperScript™III Reverse Transcriptase Kit (Invitrogen) according to the company's instructions. DNA containing each ErbB4 isoform was digested from ErbB4 isoform cloning vectors, purified in gel and serially diluted (1:5) to generate the standards. The concentration of standard DNA was estimated by measuring the absorbance at 260 nm. Standards and the unknown samples were amplified in the same plates with Taqman PCR in following conditions: 300nM of primers (Sigma), 200nM of probe, 1×Taqman



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advanced fast master mix (Applied Biosystems) and 5-10 ng templates in a 10μl reaction volume. Cycling was initiated 20s at 95°C, followed by 40 cycles of 1s at 95°C and 20s at 60°C. The probes and primers used for each isoforms are: JM-a probe [6FAM]ATGGACGGGCCATTCCACTTTACCA[BHQ1],

JM-b probe [6FAM]TTCAAGCATTGAAGACTGCATCGGCCT[BHQ1]; JM-a/JM-b forward TTGCCATCCAAACTGCACC, JM-a/JM-b reverse TCCAATGACTCCGGCTGC; CYT-1 probe [6FAM]TGAAATTGGACACAGCCCTCCTCCTG[BHQ1], CYT-2 probe [6FAM]AAGAATTGACTCCAATAGGAATCAGTTTGTGTACCA[BHQ1];CYT-1/CYT-2 forward TCCTCCCATCTACACATCCAGAA, CYT-1/CYT-2 reverse GGCATTCCTTGTTGTGTAGCAA. Gene expression data are represented as absolute copy number of each isoform per ng total RNA.

*Statistical analysis*

Data are all shown as mean ± SEM. Unless specifically indicated, data were analyzed using a one- or two-way ANOVA with Bonferroni post-hoc comparisons (Figures 2-4), or were log transformed to achieve equal variance distribution for each group before analysis via two-way ANOVA with Dunnett's multiple comparison (Figure 6). Data were considered statistically significant at $p<0.05$.

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## Results

### *3.1 Growth receptor ligands and hypertrophy development*

We sourced a range of EGF-like ligands that activate primarily ErbB1, ErbB4 or both and tested their capacity to cause hypertrophy in ventricular cardiomyocytes (Figure 1A). The most efficacious hypertrophic agents were betacellulin (BTC; maximal growth 149 ±1% compared to unstimulated), NRG1-β1 (134 ± 2%), NRG2β (134 ± 2%). Amphiregulin, TGFα, epireglin, NRG2α and NRG3 stimulated moderate levels of hypertrophy (116 ± 2, 118 ± 3, 120 ± 3, 123 ± 3, and 124 ± 3%, respectively). Hypertrophy was weak or absent when induced by EGF, HB-EGF, NRG4, epigen, and NRG1α (107 ± 2, 109 ± 2, 110 ± 3, 113 ± 2, 113 ± 3%, respectively).

Both isoforms of ERK (ERK1/2) and Akt are downstream targets of hypertrophic signalling. We therefore tested EGF-like ligands for their capacity to activate these intracellular signaling pathways. Following 5 minutes of ligand stimulation, EGF, TGFα, BTC, epiregulin and NRG1-β1induced the strongest activation of ERK and Akt (Figure 1B). Amphiregulin, NRG1α, and epigen also stimulated ERK phosphorylation, but to a lesser degree. Interestingly, while demonstrating equivalent activation of ERK and Akt, ligands were not equivalent in their ability to stimulate hypertrophy.

We next assessed the ability of BTC, NRG1-β1, NRG-2β to promote induction of hypertrophic genes and sarcomeric reorganization (both markers of cardiomyocyte hypertrophy). We examined the capacity for ErbB ligands to promote ANP gene expression using an ANP promoter-driven luciferase reporter construct (Thomas et



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al., 2002). Cardiomyocytes were transfected with both the luciferase reporter, and HA-tagged $AT_1R$ and stimulated with agonists for 48h. NRG1-β1, NRG2β and BTC induced ANP promoter activity to the same extent as AngII, whereas EGF was unable to modify promoter activity (Figure 1C).

Sarcomeric reorganization was observed via phalloidin staining of actin filaments. TRITC-labelled phalloidin was used to stain AdNHA-$AT_1$-infected cardiomyocytes (Thomas et al., 2002) stimulated with 100nM AngII, 10nM EGF, 10nM BTC, 10nM NRG1-β1 and 10nM NRG2β or left unstimulated for 72h (Figure 1D). AngII-treated cardiomyocytes developed striated actin filaments, whereas cardiomyocytes stimulated with EGF resembled unstimulated cells. In contrast, BTC, NRG1-β1 or NRG2β were able to induce actin reorganization, indicating a capacity to promote hypertrophy.

To complement our observation that EGF-like ligands, capable of activating ErbB4 receptors, are the strongest promoters of hypertrophy, we used AG1478 to pharmacologically inhibit ErbB activity, and evaluated phosphorylation of ERK (Figure 2A) and ANP promoter activity (Figure 2B). At higher concentrations of AG1478 (5μM), but not 0.5μM, both ERK phosphorylation and ANP promoter activity induced by NRG1-β1 were blocked. Consistent with previous reports that at 0.5μM AG1478 selectively inhibits ErbB1 receptors, whereas at 5μM AG1478 inhibits both ErbB1 and ErbB4 receptors (Anastassiadis et al., 2011), our data indicate a selective role for ErbB4 in NRG1-mediated hypertrophy.



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*3.2. ErbB4 is required for NRG1-β1-induced hypertrophic signaling*

Because our data mitigated against a role for direct, ligand-induced ErbB1-mediated hypertrophy, we next developed shRNA constructs that specifically knockdown ErbB2 and ErbB4 (supplementary Figure S1, A and B) and determined the effect of this knockdown on NRG1-stimulated hypertrophic gene expression, using MLC-2v, cyclin D and ANP promoter-driven luciferase reporter constructs. NRG1-induced MLC-2v, cyclin D and ANP promoter activity was significantly inhibited with knockdown of ErbB4 (Figure 2 C-E). As a corollary to our promoter-reporter assays, where only a small proportion of the myocytes were transfected with reporter and shRNA plasmid DNA (Djurovic et al., 2004; Louch et al., 2011), we also developed siRNAs that targeted ErbB2/ErbB4, because these siRNAs provide a higher knockdown efficiency in cardiomyocytes. Knockdown of ErbB2 (Figure S2, left) and ErbB4 (Figure S2, right) was observed for up to 96h after transfection. This knockdown was selective for the targeted subtype, as knockdown of ErbB2 did not affect expression of ErbB4 and vice versa (supplementary Figure S2).

NRG1-β1 stimulation increased ERK phosphorylation in cardiomyocytes (Figure 3 A and B). siRNA mediated knockdown of ErbB4 reduced NRG1-β1-induced phosphorylation of ERK by ~40% relative to the siControl (Figure 3A and B). In contrast, silencing of ErbB2 did not affect NRG1-β1-induced hypertrophic signaling (Figure 3A and B). This indicates that ErbB4 is the primary mediator of NRG1-β1-induced ERK signaling in cardiomyocytes.

We next sought to confirm that NRG1-β1 acts via the ErbB4 receptor to cause cardiomyocyte hypertrophy, as distinct from signaling activation. NRG1-β1-induced



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hypertrophic growth was significantly reduced (~ 50% compared to siControl) by down-regulation of ErbB4 and ErbB2 (Figure 3C). Consistent with this, NRG1-β1 induced sarcomere reorganization was also affected by knockdown of ErbB4 and ErbB2 (Figure 3D).

*3.3. Expression profiles of ErbB4 isoforms during physiological and pathophysiological cardiac hypertrophy*

ErbB4 has four isoforms that differ structurally in the juxtamembrane (JM) domain (JM-a and JM-b) and the intracellular CYT domain (CYT-1 and CYT-2, Figure 4A). Expression of all ErbB4 isoforms were highest prenatally and significantly decreased after birth (supplementary Figure S3), with the ratio of JM-b to JM-a increasing postnatally. Figure 4B compares expression of ErbB4 in rat ventricular cardiomyocytes isolated from P1 neonates, to that expressed in whole mouse hearts at P1 and P28. We identify dominant expression of the JM-b isoform over JM-a, and comparable expression of the CYT-1 and CYT-2 isoforms (Figure 4B).

A transgenic model of cardiac hypertrophy caused by calcineurin overexpression (Molkentin et al., 1998) was used to investigate the regulation of ErbB4 isoforms in pathological hypertrophy. Expression of all of four isoforms was significantly reduced (except JM-a, p=0.09) in calcineurin-overexpressing animals compared to the controls (Figure 4C). The JM-b:JM-a and CYT-2:CYT-1 ratios were unchanged, suggesting that the reduction of all isoforms reflects a global change in ErbB4 gene expression and not the specific splicing of individual isoforms.

*3.4. The role of ErbB4 isoforms in NRG1-β1-induced cardiomyocyte hypertrophy*



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To examine individual contributions of ErbB4 isoforms to NRG1-β1-induced cardiomyocyte hypertrophy, we inhibited endogenous ErbB4 expression using selective shRNA constructs (as detailed above) and used knockdown-resistant versions of the ErbB4 isoforms to rescue the expression of individual isoforms. Silent mutations were introduced into the ErbB4 isoforms to make them "knockdown-resistant" (kdr) to the ErbB4 shRNA (Figure 5A), and we confirmed that protein expression of kdrErbB4 receptors was not inhibited by ErbB4 shRNA 1386 (Figure 5B).

Knockdown of ErbB4 markedly reduced NRG1-β1-induced MLC-2V, ANP and cyclin D promoter activity (Figure 6 A-C). Hypertrophic responses were rescued by all four kdr-ErbB4 isoforms, indicating that the NRG1-β1 induced cardiomyocyte hypertrophy must be dependent on a common feature shared by all the isoforms (e.g. tyrosine kinase activity). One possible confounder in interpreting these data was our observation that expression of the kdr-ErbB4 isoforms led to an increase in both basal and NRG1-β1 stimulated promoter-reporter activity, suggesting that supra-physiological expression of ErbB4 receptor isoforms with constitutive activity might increase basal signalling. We were concerned that this overexpression could be masking an isoform-specific preference for NRG1-β1-mediated hypertrophy and therefore performed titration experiments with varying amounts of the kdr-ErbB4 isoforms (Figure 6D). These experiments show that no clear preference for one isoform of ErbB4 exists in relation to the NRG1-β1-mediated hypertrophic gene program.

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## Discussion

The receptors belonging to the ErbB family play an important role in the heart: abundant in cardiomyocytes, they coordinate cardiac development, physiology and growth in response to local and circulating factors, including NRG1-β1 (Parodi and Kuhn, 2014). We evaluated the capacity of ligands for ErbB receptors to induce cardiomyocyte hypertrophy, identifying a principal role for ligands that activate ErbB4, and surprisingly, a minimal role for ligands that directly activate ErbB1. NRG1-β1-induced hypertrophy was dependent on ErbB4, and may also involve ErbB2. All four ErbB4 receptor isoforms (JM-a, JM-b, CYT-1 and CYT-2) exist in heart, with the JM-b isoform predominant in terms of expression. We observed a decrease in the expression of the ErbB4 isoforms postnatally, and also in a genetic model of pathological cardiac hypertrophy (Molkentin et al., 1998). Finally, we used an RNA interference/rescue experiment to demonstrate that all four ErbB4 isoforms possess the capacity to mediate NRG1-β1 stimulated cardiomyocyte hypertrophic signaling, indicating that a common feature of ErbB4 isoforms (likely their tyrosine kinase activity) is the determinant of this function.

Our observation of potent hypertrophic effects of NRG1-β1 confirms previous reports in neonatal myocytes (Baliga et al., 1999), and studies showing NRG1-β1 improves cardiac function and prognosis for ischaemic, dilated and viral cardiomyopathy (Giraud et al., 2005; Liu et al., 2006; Rohrbach et al., 1999). Other members of the NRG family – NRG2α, NRG2β and NRG3 – also strongly stimulated hypertrophy, particularly NRG2β. The NRGs also interact with ErbB3, however, the low expression of this subtype in the adult heart (Sundaresan et al., 1998; Zhao et al.,



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1998) makes it unlikely that this receptor is mediating hypertrophy. The strongest hypertrophic agonist of all the EGF-like growth factors was BTC, which interacts with both ErbB1 and ErbB4, while also inducing heterodimerisation of these receptors with ErbB2 (Roskoski, 2014). Given that the strongest inducers of hypertrophy were BTC, NRG1-β1, and NRG2β, which all have high affinity interactions with ErbB4, the data strongly suggest that this ErbB subtype must be a critical mediator of neonatal cardiomyocyte growth.

The inability of HB-EGF or EGF, both ErbB1 ligands, to stimulate hypertrophy directly is interesting. These findings contrast studies by Asakura *et. al.* (Asakura et al., 2002) who document a >2-fold increase in [$^3$H] Leucine incorporation following HB-EGF stimulation. They also contrast with the conclusions of Yoshioka *et al.* who report a 25% increase in cell size and 34% increase in [$^3$H] Leucine incorporation with adenoviral-directed HB-EGF overexpression (Yoshioka et al., 2005). We were unable to demonstrate a direct role for HB-EGF (via ErbB1) with our hypertrophy assays (protein:DNA ratio, ANP promoter activity and cellular reorganization) up to 100 μM, despite being able to detect robust hypertrophy induction via multiple hypertrophy agents, including AngII, BTC, NRG1-β1 and NRG2β with all assays. We also observed no or little hypertrophy with other ErbB1 agonists, including EGF, amphiregulin, hTGFα, and epiregulin. Together, these data indicate that <u>direct</u> activation of EGFR (ErbB1), and very strong activation of growth signaling pathways (e.g., ERK and Akt), is not apparently sufficient to drive hypertrophy. An uncoupling of ERK/Akt activation and hypertrophy induction is consistent with previous publications by us (Kaakinen et al., 2017) and others (Kehat and Molkentin, 2010). There is also an apparent disconnect between the lack of ErbB1-mediated



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hypertrophy by direct stimulation by its ligands (e.g., EGF and HB-EGF) and the plethora of literature implicating ErbB1 in the growth of cells in response to "transactivation" by GPCRs, (Forrester et al., 2016) indicating that ErbB1 activation is necessary, but not sufficient in itself, for hypertrophy emanating from GPCRs.

ErbB2 is important in many aspects of cardiac physiology (D'Uva et al., 2015; Vermeulen et al., 2016). Interestingly, ErbB2 lacks a ligand-binding domain and thus acts in response to EGF-like factors via dimerization with other ErbB receptors and it is a preferential partner for ErbB4 (Roskoski, 2014; Wilson et al., 2009). In our studies, specific downregulation of ErbB2 using RNAi did not affect NRG1-β1-induced activation of ERK. However, NRG1-β1-induced increases in protein to DNA ratio and actin reorganization were attenuated by the knockdown of ErbB2, comparable to that seen with ErbB4 knockdown. Thus, it appears ErbB2 contributes to NRG1-β1-stimulated cardiomyocyte hypertrophy.

After identifying ErbB4 as a primary mediator of growth factor-induced cardiomyocyte hypertrophy, the focus of our study was then to evaluate the relative expression of ErbB4 isoforms in heart and their contribution to hypertrophy. These isoforms differ in their ability to be cleaved (JM-a isoforms) or not (JM-b isoforms), and to release an intracellular domain that can interact with multiple cellular factors and trigger specific signaling pathways (Ni et al., 2001; Veikkolainen et al., 2011). Furthermore, isoforms containing the CYT-1 domain have extra amino acids that interact with additional factors (e.g. YAP, STAT5, PI3K), whereas the CYT-2 domain lacks this binding site (Omerovic et al., 2004; Sundvall et al., 2008; Veikkolainen et al., 2011; Williams et al., 2004). This can lead to potentially profound functional



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differences between isoforms, as observed in the context of mammary development, astrogenesis, cancer and cell survival (Junttila et al., 2000; Muraoka-Cook et al., 2009; Sardi et al., 2006; Sundvall et al., 2010).

We observed that both the JM-a and JM-b isoforms are detectable in the adult mouse heart, although the JM-b isoform is predominant. The expression of CYT1 and CYT2 was comparable. Our observation that JM-b is the major isoform in adult heart is consistent with previous studies (Elenius et al., 1999; Elenius et al., 1997; Veikkolainen et al., 2011), although some failed to detect expression of JM-a, presumably due to limits in the sensitivity of their assays (traditional end-point PCR versus qPCR in ours). We observed that ErbB4 is expressed at a much higher level in the embryonic heart than in the adult (Figure S3). The high level of ErbB4 in embryonic heart is consistent with the strong requirement for cardiac growth during embryonic (Gassmann et al., 1995) and postnatal stages (Garcia-Rivello et al., 2005). We also characterized the cardiac expression of ErbB4 isoforms in an *in vivo* model of pathological hypertrophy with overexpression of calcineurin (Molkentin et al., 1998). All four isoforms were reduced significantly, with no selective regulation between isoforms. The decrease in ErbB4 with pathological hypertrophy is consistent with that observed for ErbB4 (and ErbB2) in failing human hearts (Rohrbach et al., 2005). One interpretation of this decrease is that ErbB4 suppression is a compensation to an over-driven hypertrophy or perhaps related to pathology, perhaps by driving proapoptotic pathways (Rohrbach et al., 2005).

To investigate the specific role of ErbB4 isoforms in hypertrophy, we developed a 'rescue' strategy using shRNA to knockdown endogenous ErbB4 isoforms, in



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conjunction with expression of knockdown-resistant (kdr) versions of individual isoforms. NRG1-β1 mediated MLC2V, ANP and Cyclin D promoter activation was abrogated by knockdown of ErbB4 and expression of each kdr ErbB4 isoform was sufficient to rescue promoter activity. In these experiments, we noted constitutive and enhanced levels of hypertrophic signaling, likely due to the supraphysiological levels of the kdr isoforms. Therefore, we titrated the amount of the knockdown-resistant constructs to see if the lower expression levels exposed selective differences in their capacity to mediate hypertrophy. No statistically significant, isoform-specific differences were observed, consistent with a common feature of ErbB4 isoforms (most likely the tyrosine kinase activity) being responsible for the ability of these receptors to mediate hypertrophic growth. It will be interesting in future experiments to use kinase-deficient mutants to confirm this assumption. A lack of an isoform-specific effect was surprising given the dominance of the JM-b isoform and the differential role of ErbB4 isoforms in other tissues (Määttä et al., 2006; Muraoka-Cook et al., 2009; Sardi et al., 2006; Sundvall et al., 2010; Zhu et al., 2006). Our data show that all ErbB4 isoforms can sustain cardiomyocyte hypertrophy, but future studies should focus on the role of ErbB4 isoforms in proliferation and regeneration. (Polizzotti et al., 2015; Vermeulen et al., 2016; Wadugu and Kuhn, 2012)

Despite the well-established role for ErbB1 in mediating transactivation via GPCRs, such as the $AT_1R$ (Forrester et al., 2016; George et al., 2013; Thomas et al., 2002), direct stimulation of ErbB1 by its ligands is insufficient to induce cardiac hypertrophy. Instead, we identified a critical role for direct ligand stimulation of the ErbB4 receptor in cardiomyocyte hypertrophy. In contrast to distinct roles for individual ErbB4



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isoform in other settings, cardiomyocyte hypertrophic signaling can be mediated equivalently by all ErbB4 isoforms, indicating that ectodomain shedding and CYT-1-directed signalling of ErbB4 do not contribute to cardiomyocyte hypertrophy.

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We thank Dr. Mona Nemer (University of Ottawa) for kindly providing the hypertrophic gene promoter luciferase reporter plasmids (MLC-2V, ANP-328, and cyclin D), Dr Andrew Dunbar (formerly of GroPep Limited, South Australia) for bovine betacelluin, Professor David Riese II (Auburn University) for neuregulin isoforms -2α, -2β, -3 and -4, and Mr. James Goonan for constructing the shRNA plasmids for ErbB2 and ErbB4. We also gratefully acknowledge cardiac tissue provided by Professor Karen Moritz (University of Queensland).

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**Figure Legends:**

**Figure 1: Role of growth factor ligands in cardiac hypertrophy development.** **A)** Rat neonatal ventricular cardiomyocytes were subjected to a titration of EGF-like ligands for 60-72 hrs. Hypertrophy assays were performed using protein/DNA lysates from cardiomyocyte preparations (n=3-4). **B)** Cardiomyocytes were stimulated with growth receptor ligands (10 nM) for 5 min and ERK and Akt activation were detected by western blot. **C)** Cardiomyocytes were stimulated with the agonists Ang II (100nM), EGF (1nM), NRG1-β1 (1nM), NRG2β (1nM) or BTC (1nM) for 72 h to induce ANP promoter activity, assessed by a luciferase reporter. **D)** The presence of sarcomeric reorganisation was detected by TRITC-labelled phalloidin staining. The white bar in the first panel is 10 μm. * $p<0.05$ vs. unstimulated.

**Figure 2. Effects of the ErbB inhibitor AG1478 on ERK phosphorylation and hypertrophic gene promoter activity in response to NRG1-β1. A)** Cells were treated with AG1478 (0.5 or 5 μM) for 30 min before stimulating with NRG1 (NRG1-β1, 10 nM) for 5 min. Levels of total and phosphorylated ERK were assessed by Western blot; images are representative of two independent experiments. **B)** Cardiomyocytes were transfected with 100 ng shControl and 300 ng ANP promoter-driven luciferase reporter constructs. 24 h later, cells were treated with AG1478 as indicated and stimulated with NRG1 (NRG1-β1, 10 nM) for 48 h. Data are presented as mean ± SEM (n=3) of relative light units normalized to the unstimulated control and analysed by two-way ANOVA with Bonferroni post-test (*$P<0.05$ vs control (no AG1478) treated with NRG1-β1). **C-E)** Cardiomyocytes were transfected with 100 ng $AT_1R$, 100 ng ErbB shRNA or shControl, and 300 ng of either MLC-2V (C), ANP (D)



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or cyclin D (E) promoter-driven luciferase reporter constructs. 24 h after transfection cells were stimulated with NRG1 for 48 h. Data are presented normalized to the NRG1-stimulated, shControl (*$P$<0.05 vs NRG-stimulated shControl) (n=3-5).

**Figure 3. Selective knockdown of ErbB receptors differentially affects hypertrophy induced by NRG1-β1.** Cardiomyocytes were transfected with siErbB2, siErbB4 or siControl and 48 h later, cells were stimulated with NRG1 (NRG1-β1, 10 nM) for 5 min. **A)** Representative western blot images showing ERK activation; **B)** Quantification of pERK/total ERK in NRG1 (NRG1-β1, 10 nM) stimulated cells (n=4). **C)** Protein:DNA ratio was determined following NRG1 (NRG1-β1, 10 nM, 48 h) stimulation and data normalized to the unstimulated, siControl (left) and **D)** Cardiomyocytes were fixed and stained with phalloidin following stimulation with NRG1 (NRG1-β1, 10 nM, 48 h). Bar = 10 μm. *$p$ <0.05 vs siControl, n=5), ‡, $p$ <0.05 vs unstimulated.

**Figure 4. ErbB4 isoform expression in cardiac tissues A)** The four isoforms of ErbB4 structurally differ in two domains: the extracellular juxtamembrane (JM) domain (JM-a and JM-b) and intracellular CYT domain (CYT-1 and CYT-2). The JM-a isoform contains 23 amino acids that allow for ectodomain shedding, while JM-b contains an alternative 13 amino acids resistant to shedding. CYT-1 is a 16 amino acid protein docking site. **B)** Absolute quantitative qPCR (TaqMan) of ErbB4 isoforms in neonatal rat ventricular cardiomyocytes and in whole hearts isolated from neonatal P1 and P28 hearts and **C)** Absolute quantitative qPCR of ErbB4 isoforms in hearts from 8 week old transgenic mice with hypertrophy due to cardiac-specific overexpression of calcineurin (hyp) and wild type controls (Cntl). Data is



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expressed as absolute copy number of each isoform per ng RNA, presented as mean ± SEM, n=3 for each time point. *, p<0.05 versus wild type controls, ‡ p<0.05 versus JM-a.

**Figure 5 Generation of shRNA knockdown-resistant (kdr) versions of ErbB4 isoforms**. **A)** schematic diagram of mutations (red, underlined) introduced into ErbB4 isoforms to make them insensitive to shRNA knockdown without altering the amino acid sequence. **B)** the kdr versions of the ErbB4 isoforms were co-expressed in COS-7 cells with ErbB4 shRNA 1386 (the shRNA targets a 21 bp sequence of ErbB4 starting at position 1386) or a control shRNA. Western blotting was used to determine ErbB4 expression.

**Figure 6: Regulation of hypertrophy gene induction by ErbB4 isoforms.** Cardiomyocytes were transfected with 100 ng of ErbB4 shRNA (shErbB4) or Control shRNA , 300 ng of luciferase reporter construct driven by **A)** MLC-2V, **B)** ANP,or **C)** cyclin D promoter and 100 ng of empty vector (v) or knockdown resistant versions of JM-a CYT-1 (a1), JM-a CYT-2 (a2), JM-b CYT-1 (b1) or JM-b CYT-2 (b2) prior to stimulation with NRG1 (NRG1-β1, 10 nM) for 48 h and determination of luciferase activity in cell extracts. (n=4-5). **C)** Cells, transfected as above, except with 20 ng, 40 ng, 60 ng, 80 ng or 100 ng of ErbB4 knockdown-resistant constructs were stimulated with NRG1 (10 nM) for 48 h (n=9). *0.05 vs. NRG1-stimulated knockdown; ‡ p<0.05 vs. unstimulated knockdown.



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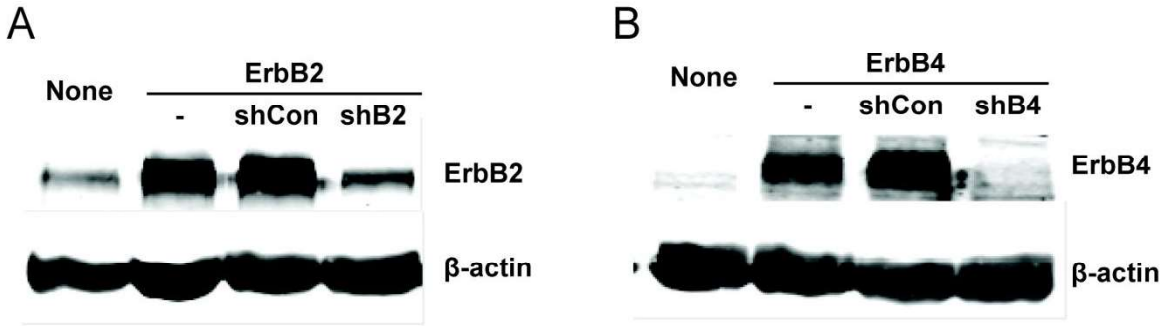
## Supplementary Information




**Supplementary Figure S1: Knockdown of ErbB2 and ErbB4 using shRNA.**

CHO cells were transfected or not (None) with 500ng of plasmids expressing either ErbB2 **(A)** or ErbB4 **(B)**, and 500ng of shRNA targeting ErbB2 (shErbB2, shB2) or ErbB4 (shErbB4, shB4), or non-targeting shRNA control (shControl, shCon) or no shRNA (-). Expression of ErbB2 **(A)** or ErbB4 **(B)** was determined by Western blot with β-actin as a loading control.

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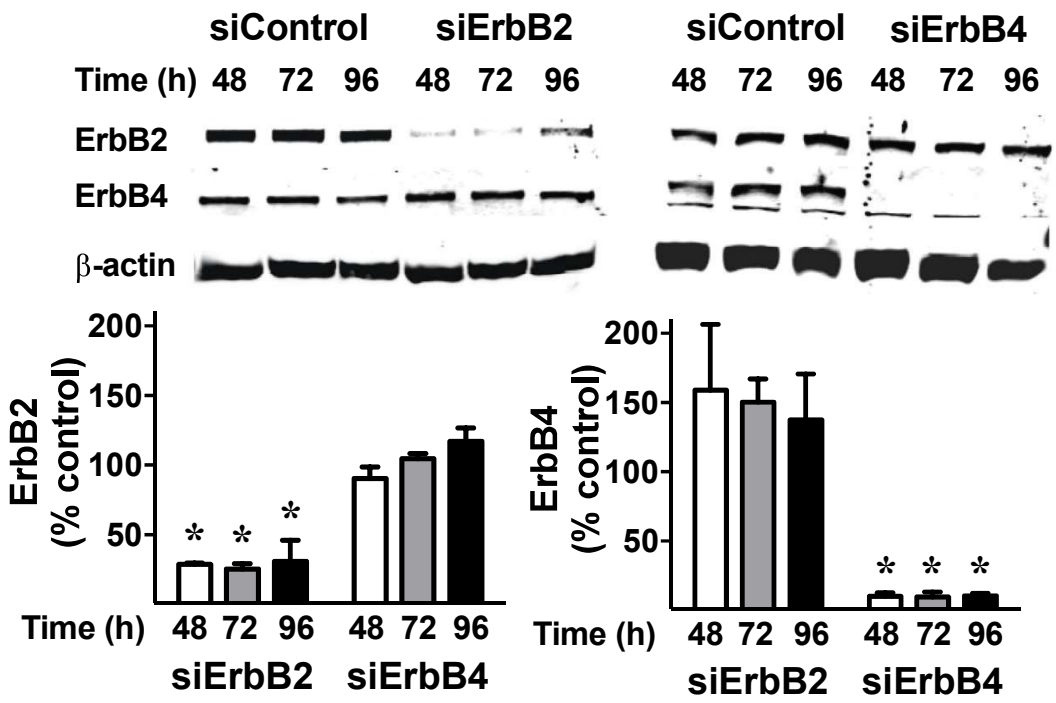


**Supplementary Figure S2: Selective knockdown of ErbB receptor expression with siRNA.** CMs were transfected with 20nM siRNA targeting individual ErbB receptors. Expression of ErbB receptors was determined via Western blotting or qPCR 48-96h after transfection. The upper images are representative images of three independent Western blots probing for ErbB2 or ErbB4. The lower graph demonstrates quantification of ErbB2 (left) and ErbB4 (right) expression using Image Studio Lite software (version 3.1). Data shown as mean ± SEM (n=3-4) and analysed by two-way ANOVA (*$p$ <0.05 vs. siControl).

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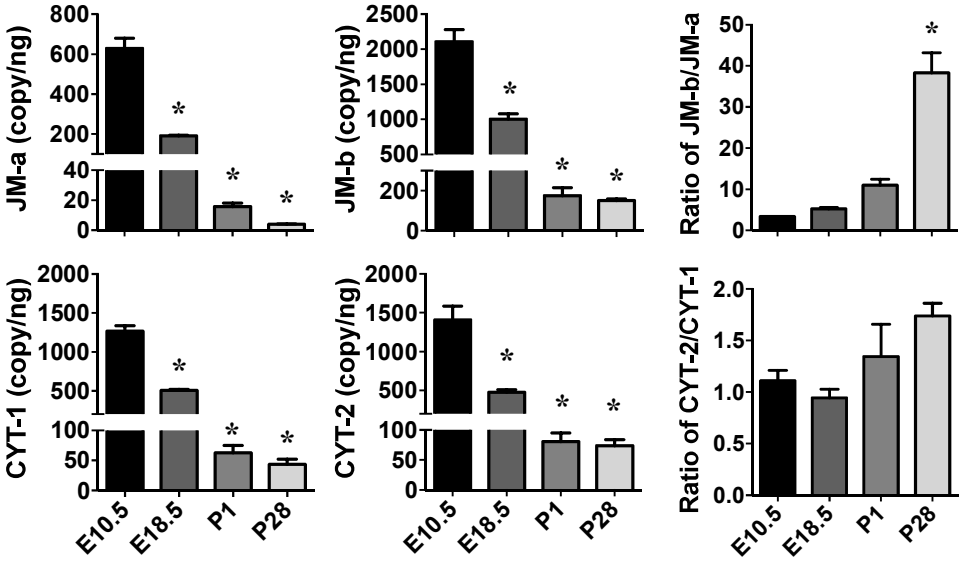


**Supplementary Figure S3: Expression profile of ErbB4 isoforms during embryogenesis and early development.** RNA was isolated from mouse hearts at embryonic day (E) E10.5, E18.5, postnatal (P) day P1 and P28 and the expression of the ErbB4 isoforms JM-a (upper left), JM-b (upper middle), CYT-1 (lower left) and CYT-2 (lower right) quantified. Ratios of JM-a/JM-b and CTY1/CYT2 (right) were also determined. Data is expressed as absolute copy number of each isoform per ng RNA, presented as mean ± SEM, n=3 for each time point. (*$p$ <0.05 vs. E10.5)

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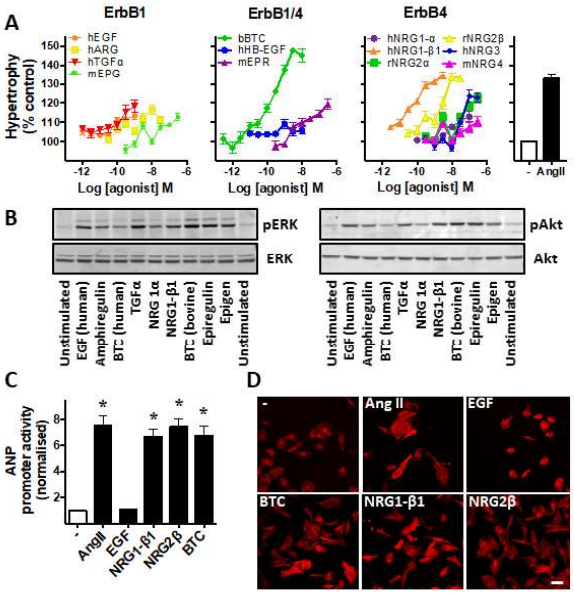


Figure 1

Figure 1 Wang et al.

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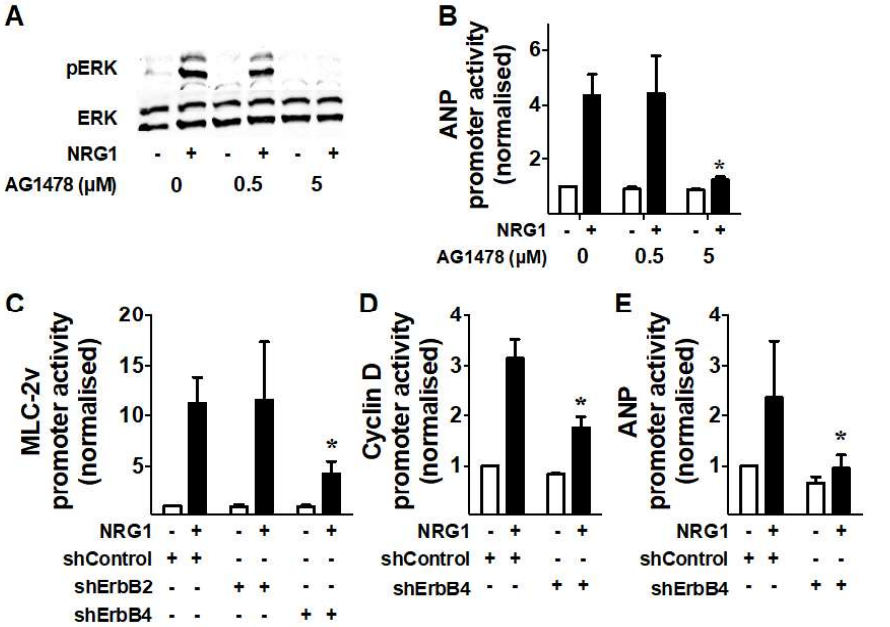


Figure 2

Figure 2 Wang et al.

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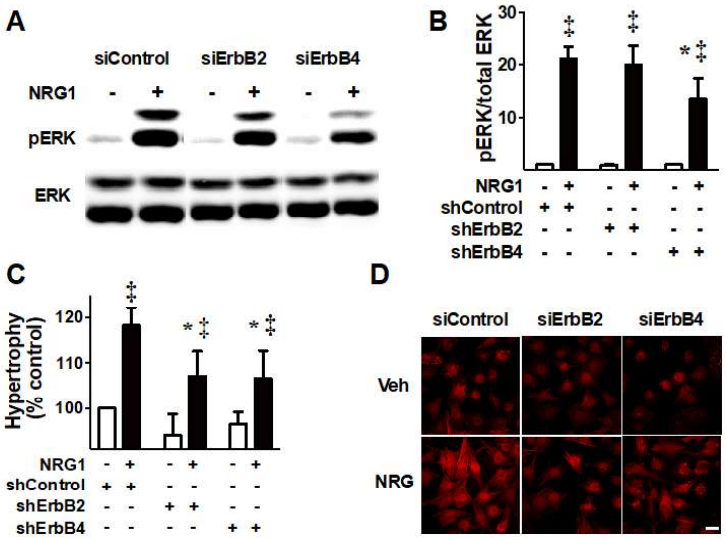


Figure 3

Wang et al. Fig. 3

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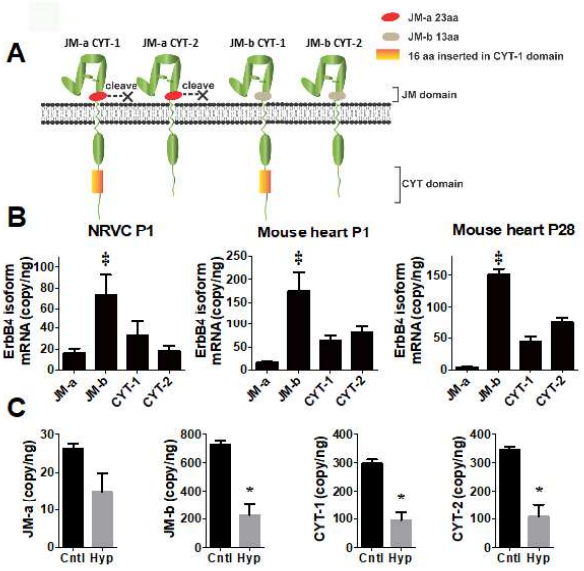


Figure 4

Wang et al. Fig. 4

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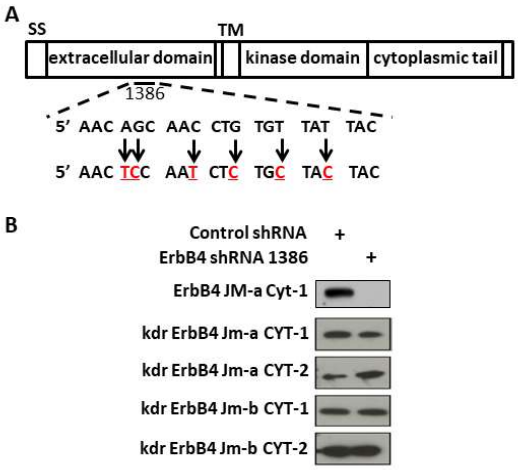


Figure 5

Wang et al. Fig. 5

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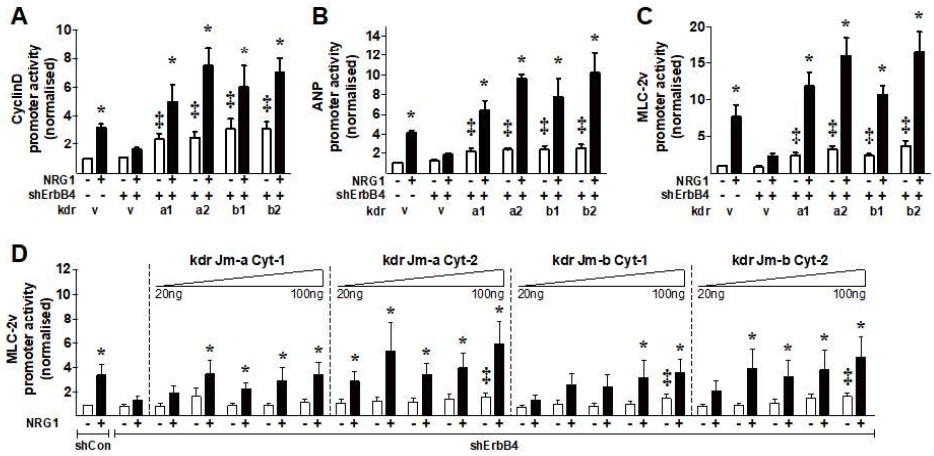


Figure 6

Wang et al. Fig. 6

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