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ErbB2 is central to the integration of multiple signaling pathways involved in cardiac function

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ErbB2 is central to the integration of multiple signaling pathways involved in cardiac function

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor in Philosophy

in

Molecular Pathology

by

Alejandra Negro

Committee in Charge:

Professor   Kuo-Fen Lee, Chair
Professor   Paul Insel, Co-Chair
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2006

**This dissertation of Alejandra Negro is approved, and is acceptable in**

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**University of California, San Diego**

**2006**

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# List of Abbreviations

| | |
|---|---|
| **ANG II** | Angiotensin II |
| **β2-AR** | Beta 2 adrenergic receptor |
| **CRFR2β** | Corticotropin-releasing factor 2 beta |
| **DCM** | Dilated cardiomyopathy |
| **EGF** | Epidermal growth factor |
| **EGFR** | Epidermal growth factor receptor |
| **Erk** | Extracellular signal-regulated kinase |
| **Glu** | Glucagon |
| **GH** | Growth hormone |
| **GPCR** | G-protein coupled receptor |
| **HB-EGF** | Heparin-binding epidermal growth factor |
| **IL** | Interleukin |
| **ISO** | Isoproterenol |
| **JAK** | Janus kinase |
| **LIF** | Leukemia inhibitory factor |
| **MAPK** | Mitogen-activated protein kinases |
| **MCK** | Muscle creatine kinase |
| **MMP** | Matrix metalloprotease |
| **NRG** | Neuregulin |

**PE**        Phenylephrine

**RTK**       Receptor tyrosine kinase

**STAT**      Signal transducer and activator of transcription

**SYF**       Src, Yes, Fyn (non-receptor tyrosine kinases)

**Ucn**       Urocortin

**VIP**       Vasoactive intestinal peptide

# List of Figures and Schema

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**Negro A**, Brar BK, Lee KF. Essential Roles of Her2/erbB2 in Cardiac Development and Function. Recent Prog Horm Res. 2004;59:1-12.

**PUBLICATIONS**

Brar BK, Jonassen AK, Egorina EM, Chen A, **Negro A**, Perrin MH, Mjos OD, Latchman DS, Lee KF, Vale W.Urocortin-II and urocortin-III are cardioprotective against ischemia reperfusion injury: an essential endogenous cardioprotective role for corticotropin releasing factor receptor type 2 in the murine heart. Endocrinology. 2004 145:24-35

Crone, SA, **Negro A**, Trumpp A, Giovannini M, and Lee KF Colonic Epithelial Expression of ErbB2 Is Required for Postnatal Maintenance of the Enteric Nervous System. Neuron 2003 37: 29-40.

## SCIENTIFIC PRESENTATIONS

**2006 Protein Phosphorylation and Cell Signaling**          August 2006
The Salk Institute for Biological Studies
"ErbB2 is required for GPCR signaling in the heart"

**10th Meeting on Protein Phosphorylation and Cell Signaling**     June 2004
The Salk Institute for Biological Studies
"Requirement of erbB2 for Integration of Multiple Signaling Pathways"

## MEETING ABSTRACTS

**Endocrine Society Meeting**                               June 2004

Urocortin-II and urocortin-III are cardioprotective against ischemia reperfusion injury: an essential endogenous cardioprotective role for corticotropin releasing factor receptor type 2 in the murine heart

# ABSTRACT OF THE DISSERTATION

ErbB2 is central to the integration of multiple signaling pathways
involved in cardiac function

by

Alejandra Negro

Doctor of Philosophy in Molecular Pathology

University of California, San Diego 2005

Professor Kuo-Fen Lee, Chair

Professor Paul A. Insel, Co-Chair

ErbB2 is a member of the epidermal growth factor receptor (EGFR/erbB1) family of tyrosine kinase receptors. Mutations resulting in the over-expression of erbB2 exhibit ligand-independent constitutive activation of its kinase domain that contributes to the pathogenesis of a variety of human cancers. The important role of erbB2 function in the adult heart was first observed in heart-specific erbB2 mutant mice (1, 2), which develop a phenotype consistent with that of dilated cardiomyopathy. The requirement of erbB2 in adult cardiac physiology was further demonstrated in clinical studies of breast cancer patients treated with Herceptin (Trastuzumab), a humanized monoclonal antibody targeting human erbB2 (Her2). A subset of these patients developed a similar cardiomyopathy to that observed in the heart-specific erbB2 mutant mice (3). In an effort to establish how the loss of erbB2-dependent signaling results in cardiac dysfunction, we developed a method for the isolation and culture of adult cardiac myocytes from wild type and erbB2 heart-specific mutant mice. In a combination of *in vitro* and *in vivo* studies, we show that erbB2 is required for key cardioprotective signaling pathways important for adult cardiac function.

The present studies confirm the concept that the erbB receptor ligand, Neuregulin (NRG), requires erbB2 for NRG-dependent signaling by demonstrating that NRG-induced activation of the extracellular signal-regulated kinase 1/2 (Erk1/2) mitogen activated protein kinase (MAPK) survival pathway is absent in heart-specific

erbB2 mutant mice. Interestingly, we show that erbB2 is not required for Erk1/2 activation in response to epidermal growth factor, another erbB receptor ligand.

We extended our studies of the requirement of erbB2 for Erk1/2 activation elicited by other classes of ligands. We established that erbB2 is critical for G-protein coupled receptor (GPCR) agonists-, Leukemia Inhibitory Factor- (LIF), and Insulin-induced Erk1/2 activation. We established that erbB2 is required for both NRG- and LIF-induced signal transducer and activator of transcription 3 (STAT3) activation, another key cardioprotective signaling pathway.

We extended our observations for the requirement of erbB2 in the cross-talk and signal integration in response to multiple classes of ligands by demonstrating that erbB2 forms a ligand-induced complex with GPCRs *in vivo* and *in vitro* and, importantly, is transactivated by a GPCR in adult mouse cardiac myocytes. We further showed that EGFR is not required for cross-talk with GPCR for GPCR agonist-induced Erk1/2 activation, as confirmed by our studies utilizing EGFR null cell lines. The ability of erbB2 to complex with multiple classes of receptors suggests a potential mechanism for erbB2 in integrating signaling networks.

# Chapter 1

# Introduction

# Her2 and Heart Failure

# HER2 AND HEART FAILURE

## ABSTRACT

The tyrosine kinase receptor erbB2, also known in humans as Her2, is a member of the epidermal growth factor receptor (EGFR or erbB1) family, which also includes erbB3 and erbB4. ErbB was discovered as an oncogene carried by an avian erythroblastosis tumor virus and exhibited similarities to human EGFR (4). Her2/erbB2 is highly expressed in many cancer types. Its overexpression is correlated with a poor prognosis for breast and ovarian cancer patients. ErbB3/4 receptors bind to a family of growth factors, termed neuregulins/heregulin (NRG/HRG), which comprise NRG-1, -2, -3, and -4 and include multiple isoforms. ErbB2/Her2 is an orphan receptor that does not bind ligand alone but heterodimerizes with the other erbB receptors for NRG signaling. ErbB2 is expressed in multiple neuronal and non-neuronal tissues in embryos and adult animals, including the heart. Genetic data demonstrated that erbB2 is required for normal embryonic development of neural crest-derived cranial sensory neurons. ErbB2/Her2-null mutant embryos of a trabeculation defect die before embryonic day (E) 11. To study its role at later stages of development, we generated a transgenic mouse line that specifically expresses the rat erbB2 cDNA in the heart under the control of the cardiac-specific {alpha}-myosin

heavy chain promoter. When crossed into the null background, the expression of the rat erbB2 cDNA rescued the cardiac phenotype in the erbB2-null mutant mice that survive until birth but display an absence of Schwann cells and a severe loss of both motor and spinal sensory neurons. To study the role of erbB2 in the adult heart, we generated conditional mutant mice carrying a cardiac-restricted deletion of erbB2. These erbB2 conditional mutants exhibited multiple independent parameters of dilated cardiomyopathy, including chamber dilation, wall thinning, and decreased contractility. Interestingly, treatment of breast cancers overexpressing erbB2 with Herceptin (TrastuzIL-ab), a humanized monoclonal antibody specific to the extracellular domain of erbB2, results in some patients developing cardiac dysfunction. The adverse effect is increased significantly in those patients who also receive the chemotherapeutical agent anthracycline. We found that erbB2-deficient cardiac myocytes are more susceptible to anthracycline-induced cytotoxicity. These results suggest that erbB2 signaling in the heart is essential for the prevention of dilated cardiomyopathy. These lines of mice provide models with which to elucidate the molecular and cellular mechanisms by which erbB2 signaling regulates cardiac functions. These mice also will provide important information for devising strategies to mitigate the cardiotoxic effects of Herceptin treatment, allowing for the potential expanded use of this drug to treat all cancers overexpressing erbB2.

**INTRODUCTION**

Cardiovascular diseases continue to be a major health risk and are the leading cause of death in both men and women (5). Dilated cardiomyopathy (DCM) is a life-threatening heart disease characterized by cardiac enlargement and decreased contractility. Gene mutations affecting cardiac myocyte contractility (e.g., sarcomeric cytoskeletal proteins) often result in the development of DCM (6). In the last decade, it was discovered that the neuregulin (NRG)/erbB signaling network plays a role in the development and function of the heart, including the formation of trabeculae (7-9), angiogenesis (10, 11), formation of heart valves (12), and prevention of DCM (1, 2). Her2/erbB2 has been a target for the development of therapeutic drugs. One major difficulty lies in the complexity of erbB2 signaling. It plays a role in multiple cell systems. Emerging evidence suggests that erbB2 is essential for transducing signaling elicited by multiple classes of ligands. Thus, it presents a great challenge to devise a selective treatment for a specific cell origin of disease. Comparative understanding of how erbB2 signals in cardiovascular and other cell systems will be important to the development of selective treatment of diseases involving deregulation of erbB2 function.

**THE ERBB RECEPTOR SIGNALING NETWORK**

NRGs play an important role in multiple cellular functions, including cell differentiation, migration, and survival. NRGs signal via ligand-induced dimerization between members of the epidermal growth factor receptor (EGFR) family of tyrosine kinase: namely, erbB2, erbB3, and erbB4. ErbB2 was identified as an oncogene due to its ability to induce the transformation of cultured fibroblasts (4). ErbB2 over-expressing cancers usually are associated with a poor prognosis and account for about 30% of metastatic breast cancer in women.

Extensive biochemical data demonstrate that NRGs preferentially bind directly to erbB3 or erbB4 that, in turn, dimerize with other erbB receptors. The erbB2 receptor does not bind directly to any known ligands and functions primarily as a co-receptor for erbB3 and erbB4. Several biochemical studies have shown that erbB2 is the preferred dimerization partner for erbB3 and erbB4 upon NRG stimulation. Receptor dimerization results in auto- and trans-phosphorylation of intracellular domains. The phosphorylated forms of these receptors then can serve as docking sites for distinct cytoplasmic proteins involved in transducing downstream signaling cascades. The tyrosine residues that are phosphorylated upon NRG binding determine the signaling molecules recruited for a particular signaling cascade (4).

Recently, two independent groups showed that the crystal structure of the erbB2 extracellular domain is in a fixed, activated conformation (13, 14). Epidermal growth factor receptor (EGFR), erbB3, and erbB4 all undergo a ligand-dependent conformational change from an inactive to an active state. The active conformation displayed by erbB2 may help explain the potent signaling effects of this orphan receptor. Due to its active conformation, other signaling molecules can use erbB2 to rapidly transduce signals without the requirement of erbB2 ligand binding (13, 14). The timing, pattern, and tissue distribution of the erbB receptors, their ligand affinity, and their ability to homo- or heterodimerize underscore the diversity of the biological response resulting from distinct NRG-induced signaling pathways in target tissues.

A great deal of erbB2 signaling research has concentrated on cancer cells, with only limited studies on the signaling of NRGs in adult cardiac myocytes. The knowledge from other cell systems will provide insights into how erbB2 might signal in adult cardiac myocytes. ErbB2 has been shown to play a role in signaling elicited by interleukin (IL)-6 in transfected prostate carcinoma cells, possibly through forming complexes with the gp130 subunit of the IL-6 receptor (15). ErbB2 is transactivated by signaling of G protein-coupled receptors (16). In human carcinoma cell lines, the integrin laminin receptors ($\alpha$6$\beta$1 and $\alpha$6$\beta$4) associate with erbB2 (17). When these cells were treated with a monoclonal antibody to the $\alpha$6 integrin, a ligand-dependent increase in erbB2 tyrosine phosphorylation was seen. Cells expressing both erbB2 and $\alpha$6$\beta$4 integrin showed enhanced proliferation rates and invasiveness, suggesting that

this receptor interaction might contribute to a more-malignant phenotype in cancer cells. In a human breast cancer cell line, known as MCF-7 cells, HRG-ß1 results in Akt activation, which is blocked by antiestrogens. HRG-ß1, bound to the erbB2/erbB3 heterodimer, in the presence of membrane estrogen receptor (ER), interacts and activates phosphatidylinositol 3-kinase (PI3-K)/Akt. Akt leads to nuclear ER/ α phosphorylation, thereby altering its expression and transcriptional activity. ErbB2 has been shown to interact with the growth hormone (GH) receptor in cancer cell lines (18). GH, by activating extracellular signal-regulated kinases (ERKs), can modulate epidermal growth factor (EGF)-induced EGF receptor (EGFR) trafficking and signaling, suggesting mechanisms of cross-talk between the GH and EGF/erbB2 signaling system.

In the heart, G protein-coupled receptor agonists such as norepinephrine, endothelin-1, and angiotensin II are known to be involved in cardiac hypertrophy via transactivation of the EGFR (19). In addition, it has been shown that a serotonin (5-hydroxytryptamine, 5-HT) mouse model that expresses inactive 5HT-2B leads to embryonic and neonatal death and a specific reduction in erbB2 and lack of trabeculae in the heart. These *in vivo* data suggest that the Gq-coupled receptor 5-HT (2B) uses the signaling pathway of tyrosine kinase receptor erbB2 for cardiac differentiation (20). In cardiac myocytes, EGFR kinase activity is involved in protease-activated receptor (PAR)4-dependent (relative of cardiac myocyte G protein-coupled receptors) activation of the p-38 mitogen-activated protein kinase (MAPK) signaling pathway,

again showing cross-talk between EGFR and G protein-coupled receptor signaling (21).

Recently, it has been shown that heparin-binding EGF-like growth factor (HB-EGF)-null mice, when viable, develop severe heart failure with grossly enlarged ventricular chambers (22). HB-EGF induces tyrosine phosphorylation of erbB2 and erbB4. The HB-EGF-null mice exhibited significantly lower levels of erbB2 and erbB4 phosphorylation, suggesting that HB-EGF acts as a cardiac survival factor partially through erbB2. Additional interactions between NRG and other signaling pathways in the heart, such as insulin-like growth factor-1 (IGF-1), have been described (23). The extent to which the NRG signaling network participates in the signal transduction of other diverse signaling pathways in the adult heart needs further investigation. Whether erbB2 associates with other G protein-coupled receptors, integrins, or gp130 in adult cardiac myocytes is unclear. It is possible that the lack of trabeculae formation in the erbB2 mutant may be due to the inactivation of a number of signaling pathways in which erbB2 functions as a central mediator.

The identification of the NRG/erbB network as potential signal integrators in response to a variety of stimuli suggests that the loss of the primary coreceptor, erbB2, either by genetic deletion (transgenic mouse models) or Herceptin treatment, cripples the coordinated cross-talk between multiple signaling pathways, resulting in loss of cellular integrity and function.

**THE ROLE OF NRG SIGNALING IN CARDIAC DEVELOPMENT**


The most-extensively studied role of NRG-mediated signaling focused on its impact on the development and maintenance of the central (CNS) and peripheral nervous systems (PNS). Knockout mice provide models with which to characterize the developmental and physiological role of a gene(s) of interest. Targeted deletion of the NRG1, erbB2, or erbB4 gene results in early embryonic lethality due to cardiac developmental defects (7-9). These murine models provided the first in vivo evidence of the role of the NRG/erbB signaling network for proper cardiac development. It is important to note that neither erbB2 nor erbB4 alone could compensate for the loss of the other receptor in the heart, suggesting that NRG signaling in the heart requires erbB2/erbB4 heterodimers. The embryonic lethal erbB3-null mice display a cardiac phenotype distinct from the NRG and NRG receptor knockout mice. The loss of erbB3 expression in the cardiac cushion mesenchyme of erbB3-null mice results in thin cardiac cushions and abnormal heart valve formation (12, 24).


**THE ROLE OF ERBB2 IN DEVELOPMENT OF HYPERTROPHY AND CELL SURVIVAL**


Both erbB2 and erbB4 receptors are expressed in isolated neonatal and adult cardiac myocytes (25). ErbB2/Her2 is localized to the transverse tubules (2) and erbB4

is localized to the caveolar microdomains within cardiac myocytes (26). *In vitro*, NRG promotes the survival of neonatal and adult cardiac myocytes and induces hypertrophic change, including changes in cell morphology, increased protein synthesis, and expression of embryonic genes (25). A soluble neuregulin-1 (NRG1, recombinant human glial growth factor 2, rhGGF2) promotes the proliferation, survival, and growth of isolated neonatal and adult cardiac myocytes. RhGGF2 provoked a two-fold increase in embryonic cardiac myocyte proliferation. RhGGF2 also promotes survival and inhibited apoptosis of serum-deprived primary cultures and induces hypertrophic growth in both neonatal and adult ventricular myocytes, which is accompanied by an increase in expression of the hypertrophic marker atrial natriuretic factor (ANF) and skeletal {alpha}-actin. NRG-1 was expressed in primary coronary microvascular endothelial cells prepared from adult rat ventricular muscle and its expression was increased by endothelin-1. This study suggested that the persistent expression of both NRG and its receptors in postnatal and adult heart affords a continuing role for the NRGs in the myocardial adaptation to physiological stress or injury. NRG-1 increased protein synthesis and induced expression of ANF and sarcomeric F-actin polymerization in neonatal rat ventricular myocytes (27). NRG-1 activated P42/44 MAPK extracellular signal-regulated kinase (ERK)-2/1 and ribosomal S6 kinase (RSK)-2, both of which could be inhibited by the ERK1-antagonist PD-098059, a MAPK/ERK kinase-1 antagonist. The NRG receptors erbB2 and erbB4 are downregulated at both the mRNA and protein levels in early stages of

heart failure in animals with chronic hypertrophy secondary to aortic stenosis, suggesting a role for disabled erbB2 signaling in the transition from compensatory hypertrophy to failure (28).

## ERBB2 SIGNALING IN ANGIOGENESIS

A study by Russell and colleagues (1999) (10) suggests an important role for NRG in promoting angiogenesis via activation of the erbB receptors in endothelial cells. ErbB2, erbB3, and erbB4 are all highly expressed in endothelial cells. In human IL-bilical vein endothelial cell (HUVEC) culture experiments, NRG stimulation results in rapid calcium influxes and cell proliferation. In vivo, NRG administration led to the growth of new blood vessels in a rat corneal angiogenesis model. Antibodies directed against vascular endothelial growth factor (VEGF), in concentrations sufficient to block high levels of VEGF, had no effect on NRG-stimulated growth or tube formation of HUVEC cells. Therefore, NRG angiogenic effects appear to be independent of VEGF (10). A subsequent study showed that Herceptin acts as an anti-angiogenic factor when administered to Her2 over-expressing breast tumors in mice (11). Herceptin also reduced the diameter and volume of tumor blood vessels, as compared to tumors treated with a control antibody. These findings expand our understanding of the anti-tumorigenic effects of Herceptin administration in the clinical setting.

# EXPERIMENTAL MODELS INVESTIGATING THE ESSENTIAL ROLE OF ERBB2

## THE ROLE OF ERBB2 IN NEURAL AND CARDIAC DEVELOPMENT

We have investigated the developmental role of erbB2 by generating erbB2-null mice (8). In this study, the mutant embryos died at embryonic day (E) 11, probably as a result of dysfunctions associated with a lack of cardiac trabeculae. Development of cranial neural crest-derived sensory ganglia was markedly affected, as was development of motor nerves. Both erbB2 and NRGs are found in the neural crest cells and migrate out of the neural tube, suggesting that both molecules act via an autocrine mechanism to play an important role in the development and differentiation of neural crest cells. Trunk neural crest cells give rise to the neuronal cells of the dorsal root ganglion, whereas cranial sensory neurons are derived from cranial neural crest cells and placodal ectoderm (29, 30).

Whole-mount immunohistochemistry with TuJ1 antibodies against neuronal tubulin revealed that the mutant embryos had no immunoreactive staining in the dorsal portion of the trigeminal ganglion or the mandibular branch of the trigeminal ganglion. The dorsal-medial portion of the trigeminal ganglion was lost in the erbB2-null embryos. There were no axonal connections between the trigeminal ganglion and the

hindbrain in sections through the whole ganglion. In mutant embryos, proximal portions of the glossopharyngeal and vagus nerves were considerably smaller than control embryos, suggesting that development of the superior and jugular ganglia was severely affected. Examination of the facial motor nerves suggested that neural crest-derived neurons in the facial ganglion also were affected. Considering the evidence of erbB2 expression in neural crest cells, the results of this study suggest that a lack of erbB2 affects the normal development of cranial neural crest-derived neurons but not placode-derived neurons at E10.5. As neural crest-derived trigeminal ganglion was markedly affected in erbB2-deficient embryos, we also investigated whether motor nerves were affected. Retrograde labeling showed that motor nerves fail to exit the hindbrain. It is likely that the development of trigeminal and facial motor nerves is secondary to a lack of the neural crest-derived portion of their respective sensory ganglia (31).

**RESCUE OF THE CARDIAC TRABECULATION IN ERBB2-NULL MICE**

Both erbB2 and NRGs are expressed in many non-neuronal tissues. In E9.5/10.5 embryos, erbB2 is detected in cardiac myocytes, whereas NRG is expressed in adjacent myocardium. This study suggested that erbB2 and NRG foster development of the heart via a paracrine mechanism. ErbB2-null mice die of a trabeculation defect before gliogenesis in the PNS (32) and before onset of neural

muscular junction at E12 (33, 34). To study the role of erbB2 after E11, our lab genetically rescued the cardiac defect in erbB2-null mice by creating transgenic mice that expressed rat erbB2 under the control of a cardiac-specific α-myosin heavy chain α-MHC promoter (35).

Mice expressing rat erbB2 in the heart were crossed with the erbB2-null mutants. The erbB2-null mutants that now over-expressed rat erbB2 in the heart formed cardiac trabeculae and survived to birth. However, due to the loss of innervation within the diaphragm muscle, the rescued mice were stillborn. Examination of the PNS in the rescued mutant embryos demonstrated a severe loss of both sensory and motor neurons and a complete absence of Schwann cell precursors in the peripheral nerves, demonstrating that erbB2 is required for normal development of the PNS (35). The prolonged viability of these mice provided further evidence supporting the important role of the NRG/erbB signaling network in the heart.

**CONDITIONAL HEART-RESTRICTED ERBB2**

The NRG/erbB receptor signaling network has been established as a key modulator/regulator of multiple developmental and physiological processes, including cardiac development and adult cardiac function. Current technology allows for the generation of mice with both temporal and tissue-specific alterations in a gene(s) of interest, referred to as conditional mutants. The conditional knockout (CKO) approach

is useful to bypass early embryonic lethality, as observed in the NRG, erbB2, erbB3, and erbB4 total knockouts, as well as to define the cell-type, tissue-specific requirements of NRG signaling in the adult.

To investigate the role of erbB2 in the adult heart, our lab generated a line of erbB2 conditional mutant mice that lacked erbB2 in the heart and skeletal muscle. To generate the mice, the erbB2 floxed allele mice were crossed to a transgenic Cre line under control of the muscle creatine kinase (MCK) promoter, a Cre line shown to promote high-efficiency recombination in atrial and ventricular lineages and postnatal skeletal muscle. A second line of heart-specific erbB2-null mice was made by breeding erbB2 floxed mice with the myosin light chain 2v (MLC2v) Cre transgenic line. The postnatal loss of erbB2 in the heart results in the progressive onset of DCM. The two independent, heart-restricted erbB2-null mouse lines display the same cardiac phenotype and differ only in the onset of DCM. Both these lines will be referred to as the erbB2 CKO mice.

The expression of erbB2 protein is significantly less in the adult hearts of erbB2 CKO mice, with no difference in the levels of the NRG receptor erbB4, compared to wild-type controls. The cardiomyopathy observed in the erbB2 CKO mice is consistent with that of dilated cardiomyopathy, including ventricular dilation, reactivation of embryonic gene expression, increase in heart:body weight ratio, and decreased cardiac contractility (**Figure 1-1**).
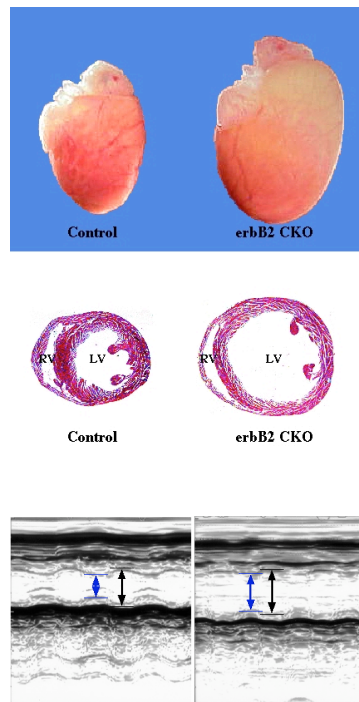
**Figure 1-1      ErbB2 conditional knockout (CKO) mice display multiple features of dilated cardiomyopathy.** (Top panel) The heart from erbB2 CKO mutants is enlarged due to dilated left ventricle (LV), as shown in hematoxylin/eosin (H/E)-stained cross-sections of the heart (middle panel). Echocardiography (bottom panel) and hemodynamic measurements demonstrate an enlarged LV with thin walls and decreased contractility. Electron microscopy reveals intact cytostructural architecture but increased mitochondria and vacuolar structures (similar to features observed following anthracycline toxicity). ErbB2 CKO cardiomyocytes are more susceptible to anthracycline toxicity in culture. [Reprinted from 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 2002 ErbB2 is essential in the prevention of dilated cardiomyopathy. Nature Med 8:459–465, with permission of the Nature Publishing Group.]

Transmission electron microscopy did not demonstrate alterations in cytoskeletal architecture but did reveal an increase in the number of mitochondria and vacuoles. The erbB2 CKO mutants have a significant increase in left ventricle (LV) end diastolic and end systolic dimensions (LVEDD and LVESD), decreased fractional shortening, decreased septal- and posterior-wall thickness, and decreased velocity of circumferential fiber shortening, compared to wild-type controls (1). In addition, retrograde catheterization of LV of the erbB2 CKO mutants revealed marked reduction in the maximum first derivative of LV pressure (LV dp/dt max), indicative of depressed myocardial contractility. No significant changes in heart rate, LV end diastolic pressure (EDP), and tau were observed between erbB2 CKO and controls. The decrease in ß-adrenergic responsiveness in the erbB2 CKO mutants are consistent with that observed in human heart failure models. However, increasing doses of the ß-adrenergic agonist, dobutamine, stimulate increased contractility and relaxation in the erbB2 CKO mice, segregating the erbB2 CKO mutants from other models of DCM. This suggests that erbB2 signaling is not required for this response. Ozcelik and coworkers (2002) (2) have independently reported similar results. Their study shows that erbB2 expression is localized to the transverse tubules of the cardiac myocytes. There was no increase in apoptosis rates in erbB2 CKO mutants. The researchers concluded that erbB2 is not required for cardiac myocyte survival but is needed for adult cardiac function.

Independently, Herceptin (Trastuzumab), a humanized monoclonal antibody directed against the extracellular domain of the Her2/erbB2 receptor, was used in clinical trials to treat female patients with metastatic breast cancer. Despite its potent ability to block erbB2-mediated signaling in breast, the cardiotoxic effects of Herceptin treatment were quite severe. This observation provided an *in vivo* link between suppression of erbB2 receptor signaling and impaired cardiac function and underscores the important role of the Her2/erbB2 receptor in adult cardiac function.

Although the majority of DCM is classified as idiopathic, it is now evident that there is a strong genetic component to the disease (36). In clinical trials for breast cancer therapy, Herceptin was an effective single agent for abolishing erbB2-mediated signaling. However, patients receiving Herceptin either following or concurrent with anthracycline treatment experienced an increased probability of developing DCM, from 7% to 28%, respectively (1). Since the majority of cancer patients have received anthracycline treatment, the effects of Herceptin treatment alone have not been fully characterized. The erbB2 CKO mice are viable and display the same cardiac dysfunction as patients treated with Herceptin. To determine whether the erbB2 CKO cardiac myocytes are susceptible to anthracycline-induced cell death, as shown in patients treated with Herceptin, neonatal cardiac myocytes isolated from the erbB2 CKO mice were with doses of Adriamycin (anthracycline) ranging from 0.5 to 5 mM. The erbB2 CKO mice displayed increased sensitivity to Adriamycin-induced

cytotoxicity. It is thus clear that the cardiotoxic effects of Herceptin are a direct consequence of the loss of erbB2 signaling in the heart.

# Chapter 2

# ErbB2 is required for GPCR agonist-induced MAPK activation

**ABSTRACT**

ErbB2/Her2, a ligandless receptor kinase, has pleiotropic effects on mammalian development and human disease. The absence of erbB2 signaling in cardiac myocytes results in dilated cardiomyopathy in mice, resembling the cardiotoxic effects observed in a subset of breast cancer patients treated with the anti-Her2 antibody, Herceptin. Emerging evidence suggests that erbB2 is pivotal for integrating signaling networks involving multiple classes of extracellular signals. However, its role in G-protein coupled receptor (GPCR) signaling remains undefined. Because the activation of the mitogen activated protein kinase (MAPK) pathway through GPCR signaling is important for cardiac homeostasis, we investigated whether erbB2 is required for GPCR mediated MAPK signaling in wild type and heart-specific erbB2 mutant mice. In order to conduct these studies under a variety of experimental conditions, we developed a mouse model of erbB2 heart-specific mutant adult cardiac myocyte cultures. This model allowed us to conduct cardiac myocyte signaling studies using erbB2 heart-specific mutants to expand our understanding of the role of erbB2-dependent signaling in adult cardiac function. These studies also helped to reveal new insights into the role of erbB2 in signal integration in response to multiple classes of ligands and the potential mechanism of such interactions with their respective receptors, observations that may later be extrapolated to other key tissues expressing erbB2. We demonstrate that erbB2, but not EGFR, is essential for MAPK activation induced by multiple

GPCR agonists in cardiac myocytes. ErbB2 is immunocomplexed with a *GPCR in vivo* and is transactivated following ligand treatment *in vitro*. Co-expression of erbB2 with GPCRs in heterologous cells results in ligand-dependent complex formation and MAPK activation. Furthermore, MAPK activation and multiple components of cardiac contractility and function *in vivo* are markedly impaired in heart-specific erbB2 mutant mice that are infused with a GPCR agonist.

**INTRODUCTION**

The erbB signaling network is a key regulator of multiple developmental and physiological processes (4, 37). Of the four members of the EGFR/erbB family, erbB2 is the preferred and potent heterodimerization partner for all erbB receptors to elicit signaling pathways, including those induced by neuregulin-1 (NRG-1) (38). Consistent with this model, the extracellular domain of the erbB2 receptor that is required for receptor heterodimerization is in a ligand-independent, activated configuration (13, 14). Thus, erbB2 is "primed" to couple with other erbB receptors for signaling. Emerging evidence suggests that it plays a pivotal role in integrating signaling networks involving other classes of extracellular signals. ErbB2 is required, for example, by interleukin-6 for signaling through the gp130 receptor in prostate carcinoma cells (15). ErbB2 may also function as a transcriptional factor (39). Hence,

genetic loss or pharmacological inhibition of erbB2 may result in an impairment of multiple signaling cascades impinging upon diverse cellular and molecular activities.

Several lines of evidence led us to investigate the role of erbB2, through GPCR signaling, in MAPK Erk1/2 activation in the heart. First, EGFR/ErbB1 has been shown to be essential in Erk1/2 activation induced by GPCR ligands in cell lines (16, 40). However, fibroblasts from EGFR mutant mice remain responsive to several GPCR ligands for Erk1/2 activation (41), raising the possibility that another tyrosine kinase receptor may be required. Interestingly, erbB2 is known to be expressed in many of the cell lines previously used for assessing the role of EGFR in GPCR signaling (see Daub et al. (16)), suggesting that erbB2 may be the required tyrosine kinase for GPCR agonist-induced Erk1/2 activation. Second, Erk1/2 plays a cardioprotective role against impaired erbB2 signaling and the cardiotoxicity observed with the chemotherapeutic agent doxorubicin, which is frequently used in conjunction with Herceptin in breast cancer patients (3, 42). Our lab has previously shown that erbB2-deficient cardiac myocytes are highly sensitive to doxorubicin (1). Heterozygous NRG-1 (NRG-1 +/-) mutant mice are more susceptible to doxorubicin-induced heart failure: Doxorubicin treatment results in decreased phosphorylated Erk1/2 levels in NRG-1 +/- mice compared to controls (43). Finally, GPCR agonists are important in cardiac homeostasis (44). For example, impaired β-AR stimulation with decreased expression and coupling of β-AR subtypes is a hallmark of heart failure (44). Doxorubicin administration to β2-AR mutant mice results in altered

Erk1/2 activation and decreased contractile function (45).  Mice over-expressing activated MEK1, an upstream activator of Erk1/2, display enhanced cardiac contractility (46). Urocortin 2, a GPCR agonist that binds to the corticotropin releasing factor receptor 2 beta (CRFR2β), is cardioprotective against ischemia by activation of Erk1/2 (47) and enhances cardiac contractility in a heart failure model (muscle-specific LIM protein-deficient mice) (48).

In the present studies, we determined the role of erbB2 in GPCR agonist-induced Erk1/2 in adult mouse cardiac myocytes isolated from wild type and heart-specific erbB2 mutant mice.  For that purpose, a mouse model of adult cardiac myocyte cultures was developed as outlined below.

## DEVELOPMENT OF ADULT MOUSE CARDIAC MYOCYTE CULTURES

Extensive studies on neonatal cardiac myocytes have provided valuable insights into cardioprotective signaling pathways in the heart, but have limited application to adult cardiac myocyte signaling due to changes in protein expression and associated myocyte developmental changes.  Primary adult myocyte cultures provide a unique model with which to study signaling in adult cardiac physiology and cardiomyopathies.  Our access to a variety of mutant mouse models generated in our lab provided the basis to establish a model with which to study cardioprotective signaling pathways in adult mouse hearts.  Therefore, we modified a protocol for the

isolation of adult rat cardiac myocytes and adapted it for use on adult mouse hearts (see **Appendix** for experimental details). We tested our protocol in a mutant mouse model lacking the class B GPCR, corticotropin releasing factor receptor 2 (CRFR2), which binds the corticotropin releasing factor (CRF) and Urocortin (Ucn) family of peptide hormones. In this initial evaluation, we showed CRFR2 is required for both Ucn 2-induced cAMP elevation (**Figure 2-1 A**) and Erk1/2 activation (**Figure 2-1 B**) (47).
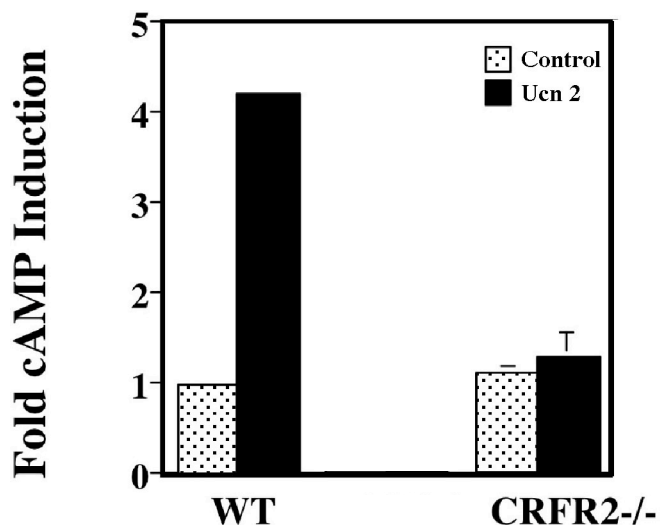
**Figure 2-1 ACRFR2 is required for Ucn 2-induced cAMP elevation.** Adult mouse cardiac myocytes were isolated from wild type and CRFR2 null mice and cultured in 12-well tissue culture dishes. Cell medium was changed from DMEM/10% (v/v) FBS to DMEM/0.1% (w/v) BSA in the presence of 1 M 3-isobutyl-1-methylxanthine (IBMX) and then exposed to Ucn 2 (10 nM) for 15 min at 37 C. Intracellular cAMP was extracted using ice cold 0.1N HCL/ 95% (v/v) ETOH. The samples were dried down under vacuum overnight. Intracellular cAMP was measured from triplicate wells using a RIA kit (Biomedical Technologies, Stoughton, MA). Forskolin (1 $\mu$M) was added to the cells to test their viability/ability to stimulate cAMP (Data not shown). Values for pmol/well cAMP were determined using Prism 3.0 software (GraphPad Inc., San Diego, CA).
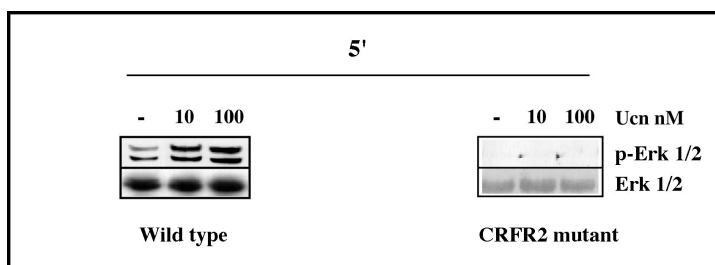
**Figure 2-1 B          CRFR2β is required for Ucn 2-induced Erk1/2 activation**
Isolated wild type and CRFR2β null adult mouse cardiac myocytes were stimulated
with serum-free media or Ucn at 10 nM or 100nM of for 5 minutes.  Cell extracts were
subjected to immunoblot analysis using an antibody that detects phosphorylated
ERK1/2 (p-ERK) and total ERK1/2 (ERK).

## RESULTS

### ErbB2 is required for Neuregulin-, but not EGFR-, induced Erk1/2 activation

Consistent with the idea that erbB2 is required for NRG-1 signaling, we found

that NRG-1-induced Erk1/2 activation is abrogated in erbB2-deficient cardiac

myocytes as compared to controls (**Figure 2-2 A**).  Interestingly, EGF induced a

marked Erk1/2 activation in both control and erbB2-deficient myocytes (**Figure 2-2

B**).

# ERBB2 IS REQUIRED FOR GPCR AGONIST-INDUCED ERK1/2 ACTIVATION

We next tested the ability of the GPCR agonist, Ucn 2, to activate Erk1/2. Ucn 2 activated Erk1/2 in controls but not in erbB2-deficient myocytes (**Figure 2-2 C**). This finding prompted us to compare the ability of three class A (angiotensin II, isoproterenol, and phenylephrine) and three class B (Ucn 2, glucagon and vasoactive intestinal peptide) GPCR agonists to activate Erk1/2 in control versus erbB2-deficient myocytes. The stimulation of Erk1/2 observed in control myocytes by all six ligands is absent in erbB2-deficient myocytes (**Figure 2-2 D**). These results demonstrate that erbB2 is required for Erk1/2 activation by multiple GPCR agonists and that EGF-mediated Erk1/2 activation does not require erbB2.

# ERBB2 IS NOT REQUIRED FOR GPCR AGONIST-INDUCED cAMP ELEVATION

In our subsequent studies, we focused on the interaction of erbB2 with $\beta$2-AR or CRFR2$\beta$. To verify that the loss of Erk1/2 activation in response to the $\beta$2-AR agonist, isoproterenol (ISO), and the CRFR2$\beta$ agonist, Ucn 2, was not due to a loss in receptor expression, we tested the ability of ISO and Ucn 2 to elevate cAMP levels in myocytes. Both ISO and Ucn 2 increased cAMP levels in control and erbB2-deficient

myocytes (**Figure 2-2 E**), indicating that erbB2-deficient myocytes express functional

β2-AR and CRFR2β. RT-PCR confirmed that β2-AR and CRFR2β mRNA levels are

not significantly different between control and erbB2-deficient myocytes (**Figure 2-3**).

In addition, both NRG-1 and EGF stimulated Erk1/2 activation in CRFR2β-deficient

myocytes (**Figure 2-4**).  In contrast, Ucn 2 fails to stimulate Erk1/2 activation in

cardiac myocytes lacking CRFR2β **(Figure 2-4)**.   These results indicate that both

CRFR2β and erbB2 are required for Ucn 2-induced Erk1/2 activation.

**Figure 2-2    ErbB2 is required for Neuregulin- and GPCR ligand-induced Erk1/2 activation in adult mouse myocardiac**    Adult cardiac myocytes were isolated from control and heart-specific erbB2 mutant mice and stimulated with NRG (5 ng/ml) (n=10, controls; n=6, mutants) (A) EGF (5 ng/ml) (n=8, controls; n=9, mutants) (B) or Ucn 2 (10 nM) (n=10, controls; n=9, mutants) (C). D, control and erbB2-difficient cardiac myocytes were untreated or stimulated with Ucn 2 (100 nM), glucagon (Glu, 100 nM), vasoactive intestinal peptide (VIP, 100 nM), angiotensin II (Ang II, 100 nM), isoproterenol (ISO, 10 µM), and phenylephrine (PE, 50 uM). Fold p-Erk1/2 induction was determined as described in Methods. E, cardiac myocytes were treated with ISO (10 µM) or Ucn 2 (100 nM) followed by measurement of cAMP levels. Fold of elevation was determined (n=5, controls; n=5, mutants). *, $p < 0.05$; ***, $p < 0.005$
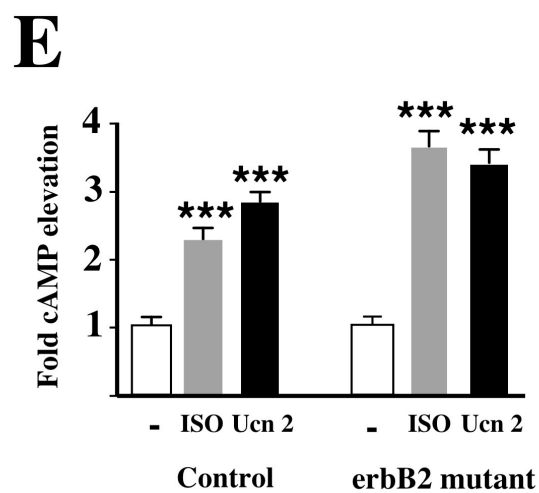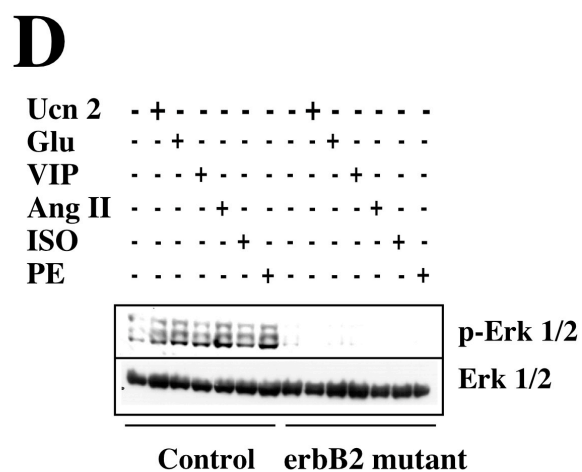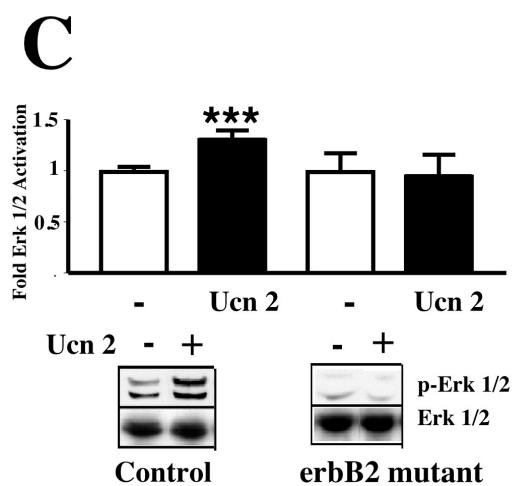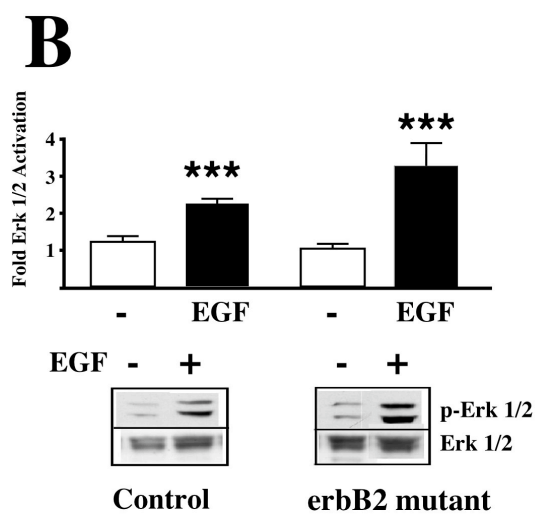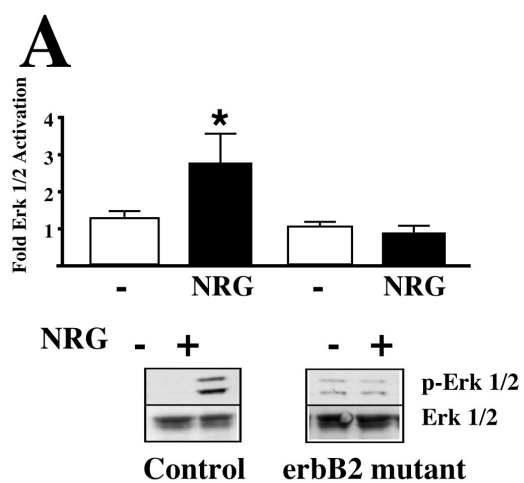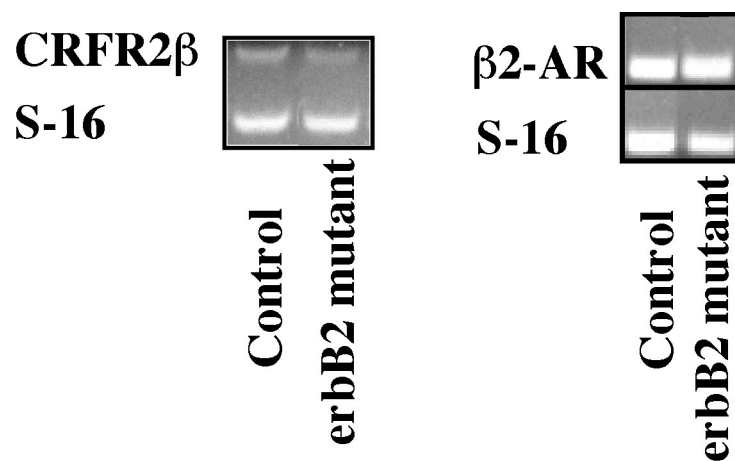
**Figure 2-3    Expression of CRFR2 and β2-AR mRNAs in the heart is not altered in heart-specific erbB2 mutants** Total RNA was isolated from controls and heart-specific erbB2 mutants and subjected to RT-PCR analysis for expression of CRFR2 and β2-AR mRNA. No differences were observed between controls and heart-specific erbB2 mutants.

NRG   -   +   EGF   -   +     Ucn2 (nM) -   10  100
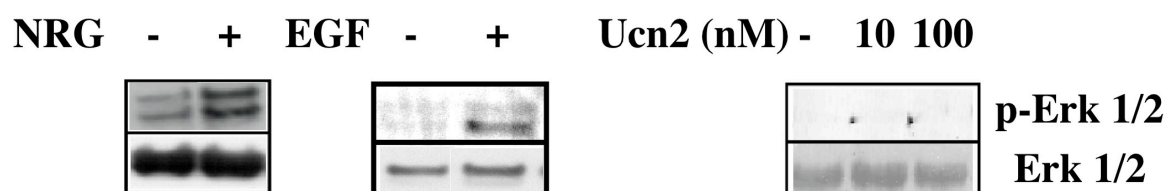


p-Erk 1/2

Erk 1/2

**Figure 2-4 Ucn 2 fails to stimulate Erk1/2 in CRFR2-deficient cardiac myocytes** Adult cardiac myocytes prepared from CRFR2 mutant mice were stimulated with NRG (5 ng/ml), EGF (5 ng/ml), Ucn 2 (10 nM or 100 nM). A representative IB of three independent experiments is shown. The results show that CRFR2 is required for Ucn 2-induced Erk1/2 activation.

# ERBB2 FORMS A LIGAND-INDUCED COMPLEX WITH A GPCR *IN VIVO* AND *IN VITRO*

The requirement of erbB2 for GPCR agonist-induced Erk1/2 activation in myocytes led us to determine whether erbB2 forms a complex with GPCRs in vivo. We found that erbB2 and β2-AR form a complex in both whole heart and brain lysates (**Figure 2-5 A**). These results suggest a potential mechanism for the requirement of erbB2 in GPCR-dependent Erk1/2 activation via complex formation of these receptors. The lack of suitable antibodies to detect endogenous CRFR2β prevented us to extend the observation of complex formation of erbB2 with CRFR2β in myocytes. However, we show that erbB2 is transactivated in response to Ucn 2 stimulation in control myocytes, suggesting that a complex formation is also likely to occur (**Figure 2-5 B**).

**A**

Heart [blot] — erbB2    [blot] — β2-AR

Brain [blot] — erbB2    [blot] — β2-AR

IP:   -β2-AR        -erbB2
IB:   -erbB2        -β2-AR

**B**

```
        -      +
erbB2 — [blot]    IP:  -erbB2
                  IB:  -p-Tyr

erbB2 — [blot]    IP:  -erbB2
                  IB:  -erbB2
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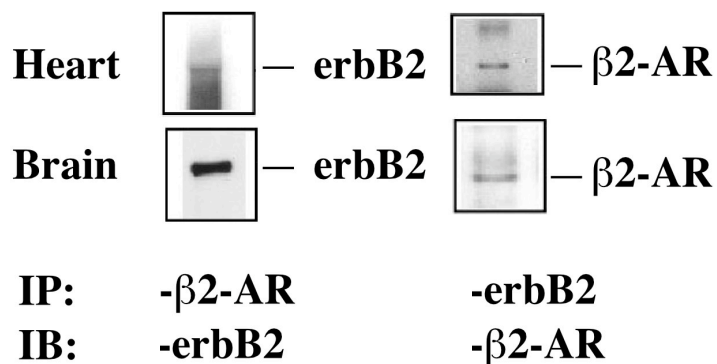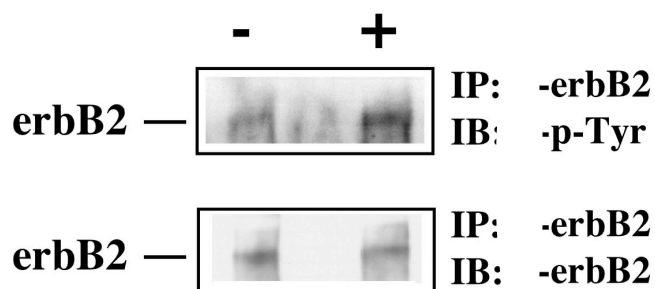
**Figure 2-5   ErbB2 forms a complex with β2-AR in whole heart and brain lystates and is transactivated by Ucn 2 in cardiac myocytes** **A,** mouse whole heart and brain lysates were incubated with either anti-erbB2 or anti-β2-AR antibodies. The resulting immune complexes were subjected to immuoblotting analysis with either anti-erbB2 or anti-β2-AR antibodies. **B,** Control cardiac myocytes were treated with serum-free DMEM (-) or Ucn 2 (100 nM).  Cell lysates were immunoprecipitated (IP) with anti-erbB2 antibodies and immnoblotted (IB) with anti-phospho-tyrosine antibodies PY-20 or anti-erbB2 antibody.

To further elucidate the mechanisms through which erbB2 and GPCRs interact to mediate Erk1/2 activation, we employed heterologous cell culture systems. When co-expressed in COS7 cells, erbB2 forms a complex with either Flag-tagged β2-AR (Flag-β2-AR) (**Figure 2-6 A**) or Flag-tagged CRFR2β (Flag-CRFR2β) (**Figure 2-6 B**). Ligand-dependent Erk1/2 activation is observed in co-transfected cells. To determine the region(s) of erbB2 required for GPCR agonist induced Erk1/2 activation, we generated tagged mutant constructs lacking either the extracellular domain (HA-erbB2ΔECD), possessing an N-terminal HA-tag, or the intracellular domain (erbB2ΔICD-Flag), with a C-terminal Flag-tag, of erbB2, as well as a kinase dead point mutant (erbB2-KD) (**See Schematic 2-1**). Heterologous cells were co-transfected with the individual erbB2 mutant constructs, together with wild type β2-AR or CRFR2β constructs, and analyzed for their ability to activate Erk1/2 in response to ligand stimulation. All three erbB2 mutants are detected on the cell surface by immunostaining (see **Appendix A1 and A2**). As shown in **Figure 2-6 C-D**, a functional kinase domain is required for both ISO- and Ucn 2-induced Erk1/2 activation. In addition, both the extracellular (**Figure 2-6 E**) and intracellular domain (**Figure 2-6 F**) of erbB2 are required for ISO-induced Erk1/2 activation. Finally, we show that wild type erbB2 is transactivated in response to ISO stimulation in cells co-transfected with erbB2 and Flag-β2-AR (**Figure 2-6 G**). The erbB2-KD mutant (**Figure 2-6 H**) and erbB2 tyrosine-to-phenylalanine, erbB2-NYPD mutant (**Schematic 2-1**), ((49)) are not transactivated by ISO when co-transfected with Flag-β2-AR (**Figure 2-6 I**).
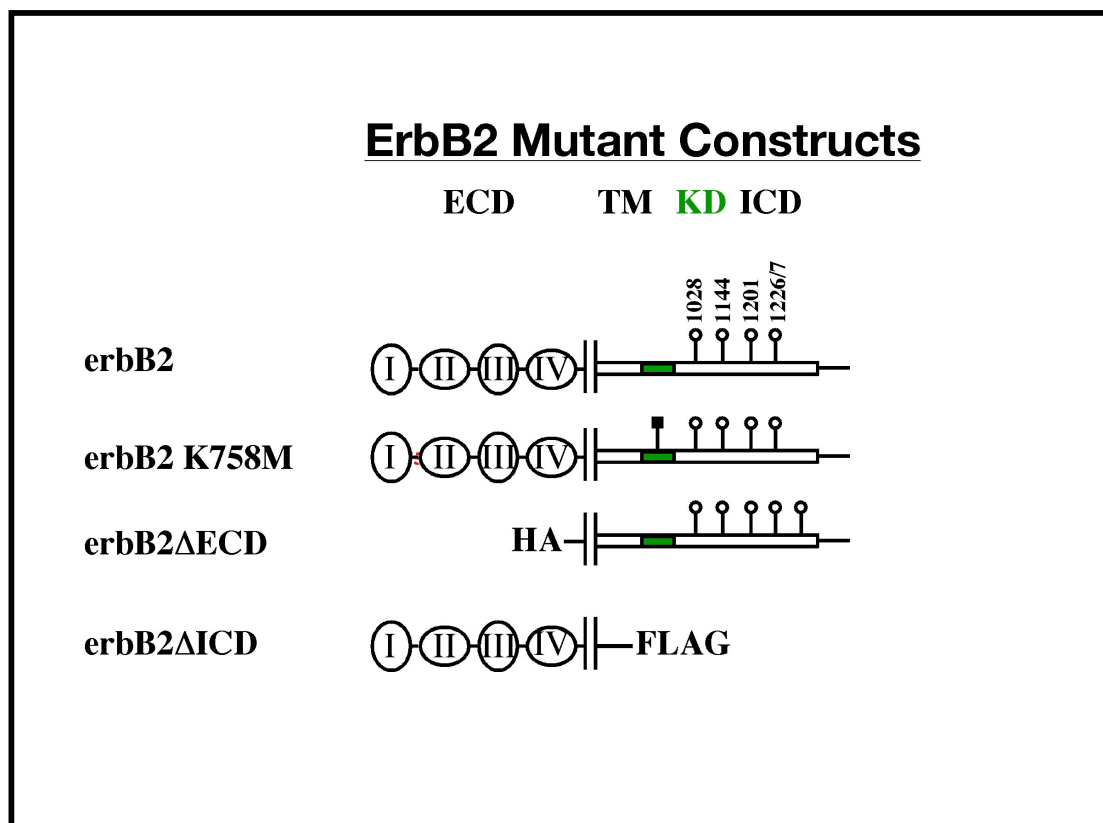
**Schematic 2-1 ErbB2 wild type and mutant constructs.**

**Figure 2-6 A-F      Ligand-dependent complex formation and Erk1/2 activation following co-expression of erbB2-AR with or CRFR2 in COS7 cells. A-B,** COS7 cells were transfected with erbB2 together with Flag-tagged β2-AR (Flag- β2-AR) (**A**) or Flag-tagged CRFR2β (**B**). Cells were treated with ISO 10 μM or Ucn 2 10 nM. Cell lysates were immunoprecipitated with α-erbB2 or α-Flag antibodies.  The IBs were probed with α-erbB2. The levels of p-Erk1/2 and Erk1/2 were measured. **C-D**, COS7 cells were transfected with Flag- β2-AR (c) or Flag-CRFR2β with erbB-kinase dead (erbB2-KD) (**C-D**). **E-F**, COS7 cells were transfected with Flag- β2-AR along with HA-erbB2ΔECD (**E)** or erbB2ΔICD-Flag (**E**) Cells were treated with ISO 10 μM and cell lysates were immunoprecipitated with α-erbB2 or α-Flag antibodies. The IBs were probed with α-erbB2. The levels of p-Erk1/2 and Erk1/2 were also measured.

**A**

pcDNA3    + + + + + + - - - -
ErbB2     - - + + - - + + + +
Flag-β2-AR - - - - + + + + + +
ISO       - + - + - + - + - +

IB:   IP Lysate ————— ErbB2

IP:   α-Fl   α-B2   α-Fl   α-B2

p-Erk 1/2
Erk 1/2

IB:   Total Lysate ————— ErbB2

**B**

pcDNA3       + + + + + + - - - -
ErbB2        - - + + - - + + + +
Flag-CRFR2β  - - - - + + + + + +
Ucn 2        - + - + - + - + - +

IB:   IP Lysate ————— ErbB2

IP:   α-Fl   α-B2   α-Fl   α-B2   α-Fl

p-Erk 1/2
Erk 1/2

IB:   Total Lysate ————— ErbB2

**C**

pcDNA3            + + + + + + - -
Flag-β2-AR        - - + + - - + +
ErbB2-Kinase Dead - - - - + + + +
ISO              - + - + - + - +

p-Erk 1/2
Erk 1/2

IB:   Total Lysate ————— ErbB2

**D**

pcDNA3             + + + + + + - -
Flag-CRFR2β        - - + + - - + +
ErbB2-Kinase Dead  - - - - + + + +
Ucn 2             - + - + - + - +

p-Erk 1/2
Erk 1/2

IB:   Total Lysate ————— ErbB2

**E**

pcDNA3      + + + + - - - -
Flag-β2-AR  - - + + + + + +
ErbB2ΔECD   - - - - + + + +
ISO        - + - + - + - +

p-Erk 1/2
Erk 1/2

IB:   Total Lysate ————— ErbB2ΔECD

**F**

pcDNA3      + + + + - - - -
Flag-β2-AR  - - + + + + + +
ErbB2ΔICD   - - - - + + + +
ISO        - + - + - + - +

p-Erk 1/2
Erk 1/2

IB:   Total Lysate ————— ErbB2ΔICD

**Figure 2-6 G-I ErbB2 is transactivated *in* *vivo* by ISO.** COS7 cells were transfected with Flag-tagged β2-AR (Flag-β2-AR) together with wild type erbB2 (**G**), erbB-kinase dead (erbB2-KD) (**H**), or the erbB2 tyrosine mutant, NYPD (**I**). Cells were stimulated with serum free media (-) or with ISO (10 μM) for five minutes. Cell lysates were immunoprecipitated with anti-erbB2 or anti-Flag antibodies and probed with an antio-phosphotyrosine antibody, PY20. Blots were stripped and probed with anti-erbB2, to confirm that the PY20 antibody was detecting erbB2.

**G**
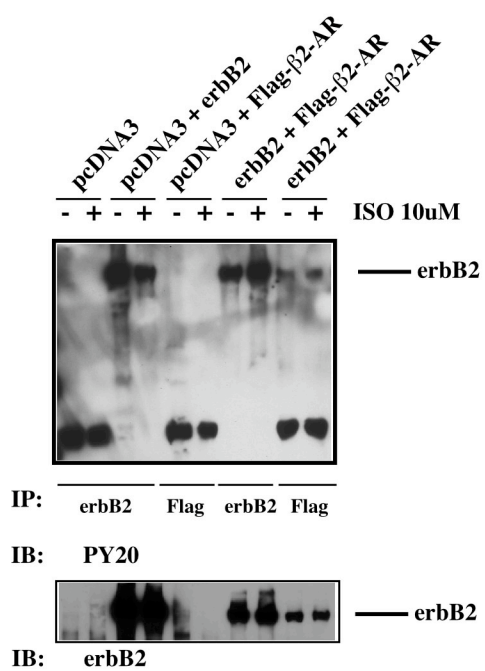


**H**
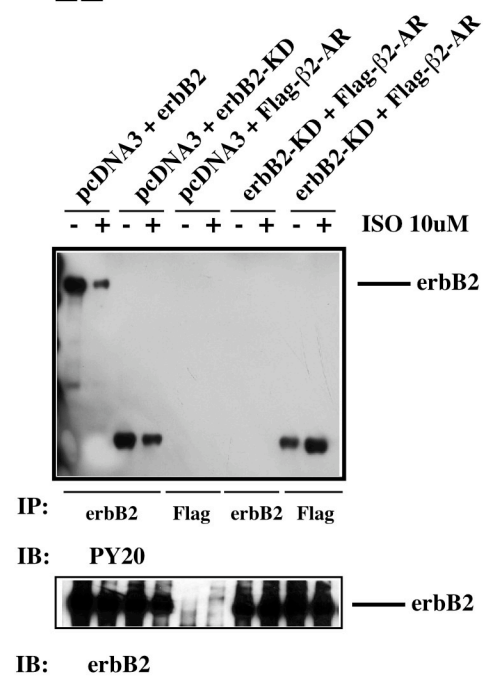


**I**
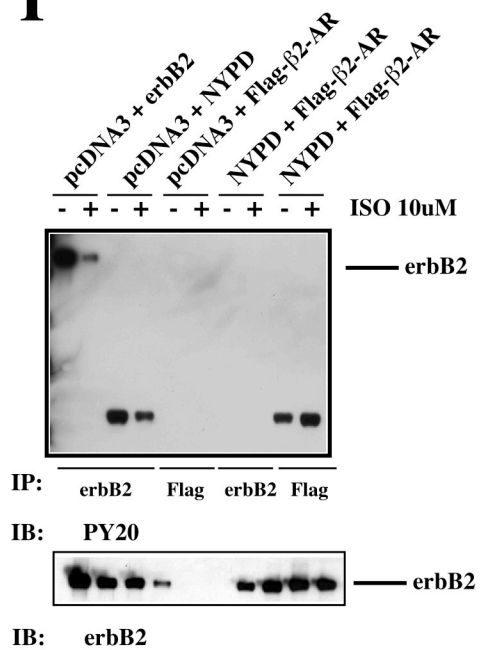
**EGFR IS NOT REQUIRED FOR GPCR AGONIST-INDUCED ERK1/2 ACTIVATION**

To determine whether EGFR is required for GPCR-mediated Erk1/2 activation, we tested the ability of ISO, Urocortin 1 (Ucn 1), and Ucn 2 to activate Erk1/2 in B82L cells that lack endogenous EGFR and express erbB2 (50), $\beta$2-AR and CRFR2, as shown in **Figure 2-7**. We found that ISO, Ucn 1 and Ucn 2 (Ucn 1 and 2 bind CRFR2) are capable of activating Erk1/2 in the absence of EGFR (**Figure 2-8 A**). In addition, clinical trials using human EGFR kinase inhibitors for treatment of cancer report no negative impact on cardiac performance, suggesting that EGFR-dependent signaling in the heart is not essential, at least for the duration of the trial (51), further supporting the notion that EGF/EGFR are not essential for adult cardiac homeostasis . However, we cannot rule out the possibility that EGFR may play some role facilitating and/or modulating erbB2-GPCR cross talk in response to GPCR agonist-induced Erk1/2 activation, including the pathway involving GPCR-mediated release of soluble ligands for EGFR (52, 53).

# SRC, YES, AND FYN ARE NOT REQUIRED FOR GPCR AGONIST-INDUCED ERK1/2 ACTIVATION

The Src family of non-receptor tyrosine kinases, which includes Src, Lyn, Fyn, Fgr, Yes, Lck, Blk, and Hck, are important mediators of multiple physiological processes, such as cell proliferation, survival, and adhesion (54). Members of the Src non-receptor tyrosine kinase family have also been shown to be important for GPCR-agonist induced Erk1/2 activation via EGFR (40).  To test this possibility, we obtained a mouse embryonic cell line, SYF, lacking three non-receptor tyrosine kinases; src, yes, and fyn (41) and that also expresses $\beta$2-AR (**Figure 2-7**).  We found that ISO activates Erk1/2 in non-transfected SYF cells (**Figure 2-8 B**), consistent with data from Huang et al (55).  Ucn 2 does not activate Erk1/2 in non-transfected SYF cells because they do not express CRFR2$\beta$ (**Figure 2-7**).  Co-transfection of erbB2 and CRFR2$\beta$ in SYF cells resulted in Erk1/2 activation in response to Ucn 2 (**Figure 2-8 C**).  Taken together, these results indicate that src, yes and fyn are not required for GPCR agonist-induced Erk1/2 phosphorylation via erbB2.  These results suggest that other cellular kinases and/or scaffolding molecules are required for erbB2 to mediate GPCR ligand-induced Erk1/2 activation (56, 57).
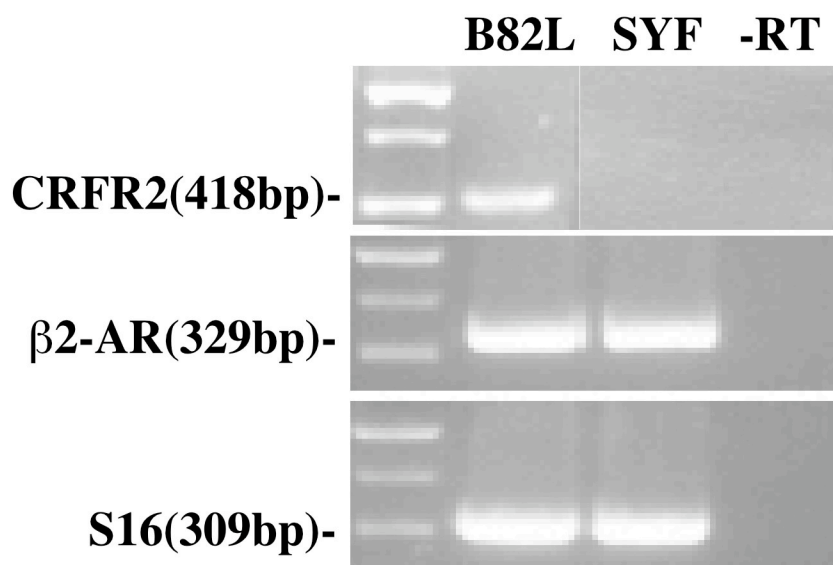
**Figure 2-7      Expression of CRFR2β and β2-AR mRNAs in B82L and Src, yes and Fyn deficient cells (SYF) fibroblasts** Total RNA was isolated from B82L and SYF cells and subjected to RT-PCR analysis for expression of CRFR2 and β2-AR mRNAs. Both CRFR2 and β2-AR mRNAs were expressed in B82L cells. Only β2-AR mRNA was detected in SYF cells.
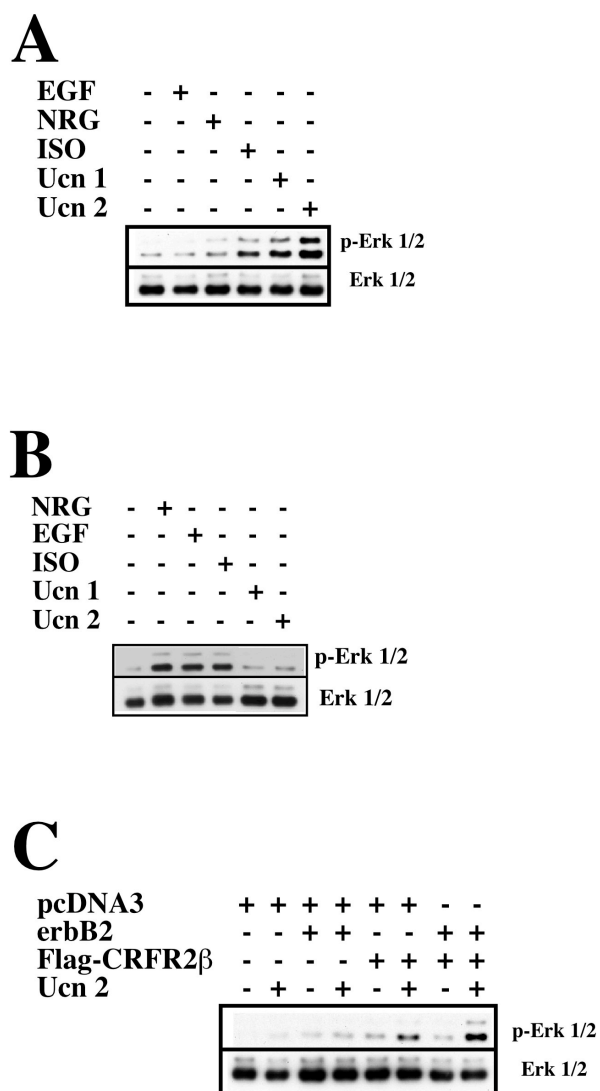
**Figure 2-8    EGFR and non-receptor tyrosine kinases src, yes and fyn are not required for ISO- and Ucn 2-induced Erk1/2 activation.** B82L EGFR-deficient fibroblasts were stimulated with serum-free DMEM, NRG (5 ng/ml), EGF (5 ng/ml), ISO (10 μM), Ucn 1 (10 nM) or Ucn 2 (10 nM).  Phosphorylated Erk1/2 (p-Erk1/2) and total Erk1/2 levels were determined. **B**, SYF cells were stimulated with serum-free DMEM, NRG (5 ng/ml), EGF (5 ng/ml), ISO (10 μM), Ucn 1 (10 nM) or Ucn 2 (10 nM).  **C,** SYF cells were transfected with pcDNA3, pcDNA3 plus erbB2, pcDNA3 plus Flag-CRFR2β, or erbB2 and Flag-CRFR2β.  Cells were stimulated with serum-free DMEM, Ucn 2 (10 nM).

## β-ARRESTIN 1 AND 2 ARE NOT REQUIRED FOR GPCR AGONIST-INDUCED ERK1/2 ACTIVATION

To date, multiple mechanisms of GPCR-mediated activation of MAPK have been proposed, including recruitment of β-arrestin, G-protein mediated activation of the non-receptor tyrosine kinase, Src, and transactivation of tyrosine kinase receptors. Although a primary function of β-arrestins are to bind phosphorylated GPCRs and target these receptors for endocytosis, they have also been shown to be involved in MAPK activation (55). Consistent with their established role in receptor endocytosis, genetic deletion of β-arrestin 2 or β-arrestin 1 and 2 in mouse embryonic fibroblasts results in the loss of β2-AR internalization in response to agonist stimulation (55). However, as in the case of the SYF null cells (**Figure 2-8 B-C**), the loss of β-arrestin has no impact on ISO-induced MAPK activation (**Figure 2-9**), consistent with that observed by Huang et al (2004) (55).
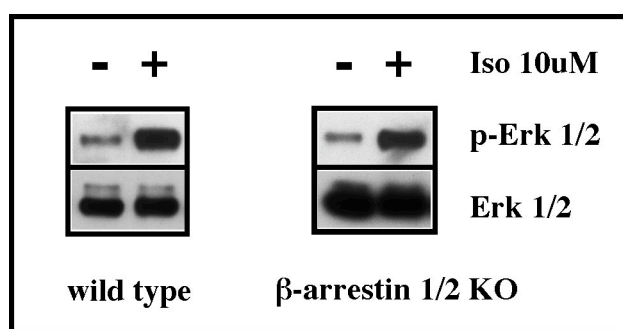


**Figure 2-9** **β-arrestin 1 and 2 are not required for ISO-induced Erk1/2 activation** Wild type and β-arrestin 1/2 double knock-out mouse embryonic fibroblasts were serum starved overnight and then stimulated with serum free media or ISO (10 μM) for 5 minutes. Cell lysates were immunoblotted for p-Erk1/2, followed by Erk1/2.

# *IN VIVO* CORRELATES OF ERBB2-GPCR INTERACTIONS

# ERBB2 IS REQUIRED FOR UCN 2-INDUCED ERK1/2 ACTIVATION AND ITS INOTROPIC EFFECTS *IN VIVO*

To determine whether erbB2 is required for the *in vivo* activation of Erk1/2 in the heart, wild type and heart-specific erbB2 mutants were studied prior to and after infusion with Ucn 2. There were no significant differences between basal levels of Erk1/2 in wild type and heart-specific erbB2 mutants. Administration of Ucn 2 to wild type mice resulted in a significant increase in Erk1/2 activation. In contrast, infusion of Ucn 2 to heart-specific erbB2 mutant mice did not result in an elevation of Erk1/2 phosphorylation (**Figure 2-10 A**). To determine the effects of the loss of erbB2 in cardiac myocytes, the physiological effects of Ucn 2 were assessed by cardiac catheterization in both wild type and heart-specific erbB2 mutant mice. In comparison to wild type littermates, the responsiveness of left ventricular peak dP/dt (a relatively specific measure of contractility), ejection fraction, stroke work, and cardiac output following Ucn 2 infusion were all significantly decreased in the heart-specific erbB2 mutant mice (**Figure 10 B-E**). There were no differences in the response to Ucn 2 in heart rate or aortic elastance in wild type versus heart-specific erbB2 mutant mice (data not shown). Overall, our *in vivo* physiologic measurements suggest that the absence of erbB2 abrogates the activation of Erk 1/2 by Ucn 2 and mitigates the potent enhancement of left ventricular function by this GPCR agonist.

These results suggest that erbB2 is required for mediating the contractile response of a

GPCR agonist, such as Ucn 2, *in vivo*, thus providing an *in vivo* correlate to the *in vitro*

observations described above.

**A**
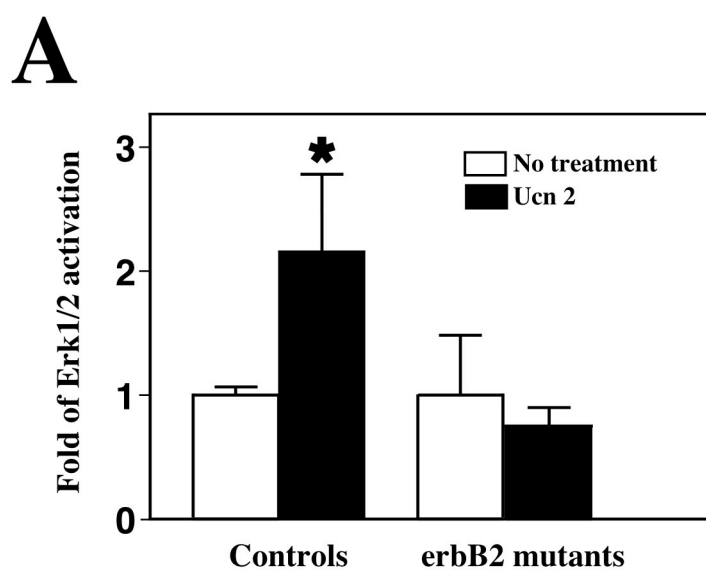


**Figure 2-10   ErbB2 is required for Ucn 2-induced Erk1/2 activation *in vivo* and left ventricular responsiveness   A**, Control and heart-specific erbB2 mutants were untreated or infused with Ucn 2 (1.0 mg/kg).  Levels of p-Erk1/2 and total Erk1/2 in heart lysates were determined.

**Figure 2-10 B-E     ErbB2 is required for Ucn 2-induced Erk1/2 activation *in vivo* and left ventricular responsiveness** Hemodynamic analysis of mice following Ucn 2 infusion. Peak dp/dt (**B**), left ventricle ejection fraction (**C**), left ventricle stroke work (**D**) and cardiac output (**E**) following administration of Ucn 2 to control and erbB2 mutant mice was determined by cardiac catheterization. Indices of left ventricular function (mean + SEM), recorded in anesthetized basal state and in response to intravenous bolus administration of Ucn 2. Note that responsiveness of these indices to Ucn 2 is significantly diminished in the erbB2 mutant mice as compared to their control littermates.

**B**



**C**



**D**



**E**
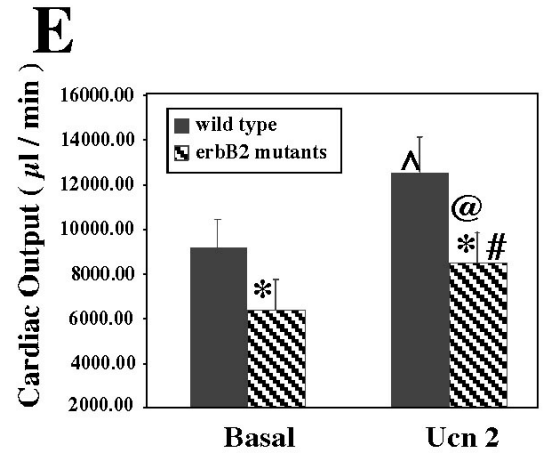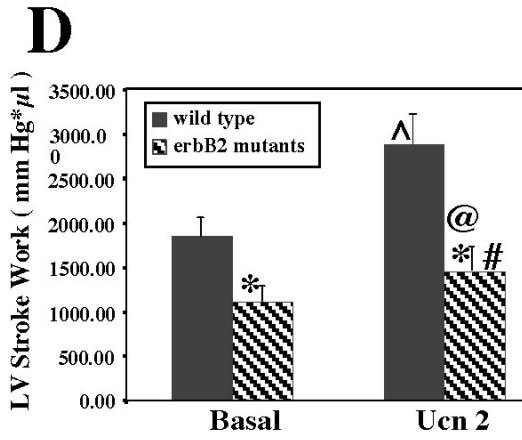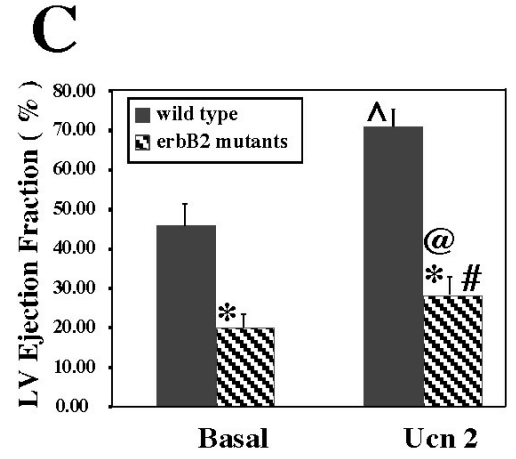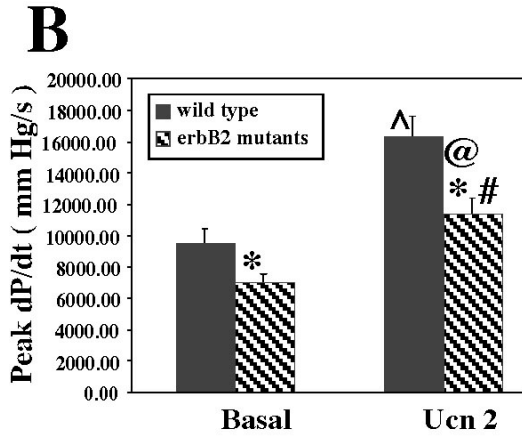
**DISCUSSION**

The present study provides evidence that erbB2 is required for Erk1/2 activation induced by multiple GPCR ligands in the heart (**Figure 2-2 E**). We propose a model in which erbB2 forms a complex with GPCRs in a ligand-dependent fashion followed by transactivation of erbB2 and activation of downstream signaling cascade(s) leading to Erk1/2 activation.

We show that while EGF activates Erk1/2 in erbB2-deficient cardiac myocytes, (**Figure 2-2 B**) EGFR is dispensable for GPCR agonist-induced Erk1/2 activation, as shown in B82l EGFR null cells (**Figure 2-8 A**). The loss of GPCR ligand-induced Erk1/2 activation in erbB2-deficient myocytes in the presence of EGF/EGFR signaling implicates erbB2 as the required receptor tyrosine kinase (RTK) involved in a potential RTK/GPCR heterocomplex in cardiac myocytes. Thus, the results expand the earlier observation by Andreev et al (41) showing that EGFR was dispensable for MAPK activation in response to GPCR agonist stimulation in EGFR-null mouse embryonic fibroblasts.

The data also show that β-arrestin 1 and 2 are not required for GPCR-agonist induced Erk1/2 activation (**Figure 2-9**), as also shown by Huang et al (55). The use of cells isolated from genetic knock-out mouse models of EGFR, Pyk2, Src, and β-arrestin provide the strongest evidence that another tyrosine kinase receptor, likely

another member of the erbB receptor family, may be involved in mediating the ERK1/2 response.

It will be of interest to test our model with additional GPCR ligands in different cell types where erbB2 is expressed to extend our observation to other key cellular systems. For example, we showed that erbB2 forms a complex with β2-AR in the brain (**Figure 2-5 A**), where other classes of GPCR type neurotransmitter receptors and erbB2 are expressed and where the Erk1/2 signaling cascade is a key regulator of pathways for cell survival and synaptic function (58). The interaction of erbB2 and GPCRs may regulate different aspects of cancer progression, in tumors such as prostate, breast and non-small cell lung cancers, where these receptor families have been shown to play a role (59, 60). Finally, we show that erbB2 is also required for signaling induced by NRG-1 (**Figure 2-2 A**), leukemia inhibitory factor and insulin in cardiac myocytes (**see results in Chapter 3**), suggesting that erbB2 has distinct roles in mediating MAPK activation in response to a variety of ligand and receptor partners. Our results uncovered an essential role of erbB2 in receptor tyrosine kinase -GPCR cross talk, consistent with the idea that erbB2 is required for the integration of diverse, key signaling pathways.

**METHODS**

**ADULT CARDIAC MYOCYTE CULTURES AND STIMULATION**

Adult mouse cardiac myocytes were isolated from three to six month old controls, heart-specific erbB2 mutants (1), or CRFR2β mutants (48) as described previously (47) (See Appendix for experimental details). Cells were stimulated with Ucn 2 (100 nM), glucagon  (100 nM), vasoactive intestinal peptide (100 nM), angiotensin II (100 nM), isoproterenol (10 μM), and phenylephrine (50 mM) for 5 min or 5 ng/ml NRG-1 or EGF for 15 min.  Levels of p-Erk1/2 and total Erk1/2 (Erk1/2) (Santa Cruz) were measured by immunoblotting analysis using specific antibodies against p-Erk1/2 and Erk1/2, respectively.  Fold increase of p-Erk1/2 was expressed as mean+s.e.m. Single-factor one-way ANOVA was performed for each group of treatments.  Differences among means were compared within the treatment groups using the Student's t test.  Intracellular cAMP levels were measured by a radioimmunoassay as described previously (47).

**TRANSIENT TRANSFECTION AND IMMUNOPRECIPITATION**

COS7, B82L and SYF cells were transfected with pcDNA3 or various expression plasmids. For immunoprecipitation, cells and tissues were lysed with RIPA buffer, pre-cleared by incubation with protein A/G-agarose beads and immunoprecipitated with anti-

erbB2 (C-18, Santa Cruz; AB3 and AB4, Oncogene), anti-β2-AR (H-20, Santa Cruz) or anti-Flag antibodies. Tyrosine phosphorylated erbB2 levels were determined by immunoblotting analysis using anti-phospho-tyrosine antibodies PY20.

## *IN VIVO* UCN 2 SIGNALING AND HEMODYNAMIC ANALYSIS

Left ventricular function in response to Ucn 2 (1 µg/kg i.v.) was assessed during general anesthesia by catheter micromanometry and volumetry in 9 month old controls and heart-specific erbB2. Hearts were frozen under liquid $N_2$ and homogenized with 1 ml of ice cold Tris-maleate (pH 7.0) buffer containing 0.2 M sucrose, 2 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 10 µg/ml aprotinin and 0.1 mM 3-isobutyl-1-methylxanthine.

# Chapter 3

# ErbB2 is required for Leukemia Inhibitory Factor- and Insulin-induced Erk1/2 activation

**ABSTRACT**

ErbB2 has been shown to act as a mediator for cytokines, and forms complexes with the gp130 subunit of the IL-6 receptor in transfected prostate carcinoma cells (15). Cytokines play a critical role in the development and homeostasis of many organ systems. In particular, the Interleukin-6 (IL-6) family of cytokines, which includes IL-6, IL-11, leukemia inhibitory factor (LIF), cardiotrophin-1 (CT-1), ciliary neurotrophic factor (CNTF), oncostatin M (OSM), and cardiotrophin-like cytokine (CLC) has been implicated in both normal and pathological cardiac growth. IL-6 cytokines require the gp130 signal transducing subunit for activation of downstream targets, which is expressed in multiple cell types throughout development and in the adult. The binding of IL-6 cytokines to their respective receptor complexes results in the tyrosine phosphorylation of gp130, which is dependent upon intracellular tyrosine kinases, since gp130 itself has no intrinsic tyrosine kinase activity (61).

Based on the important role of erbB2 and IL-6 cytokine signaling components in cancer and cardiac physiology, I sought to test if erbB2 is required for LIF-induced STAT3 and Erk1/2 activation in adult mouse cardiac myocytes.

**INTRODUCTION**

Mice harboring a targeted deletion of gp130 die in utero due to multiple organ defects, including thin-walled and dilated cardiac ventricles, suggesting this signaling pathway is critical for proliferative cardiac growth (62). Mice lacking gp130 in ventricular myocytes develop normally and display no overt cardiac phenotype (63). However, after proximal aortic banding, the heart-specific gp130 mutant mice developed marked cardiac abnormalities, including the rapid development of dilated cardiomyopathy and an increase in myocyte apoptosis, which is associated with a loss of STAT3 phosphorylation. These studies indicate that gp130-mediated signaling is necessary not only for normal cardiac development, but is also crucial for cardiac myocyte survival in conditions of compensatory hypertrophy.

IL-6 cytokine signaling via gp130 mediates cardiac myocyte survival and hypertrophy via the janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) and Erk1/2 MAPK pathways. JAKs are intracellular, non-receptor tyrosine kinases that are essential for mediating cytokine signaling. Cytokine receptor activation in response to ligand binding results in JAK auto- and transphosphorylation of the cytokine receptor complex and gp130, which then provides docking sites for the recruitment of STATs (64).

STATs are latent, cytoplasmic transcription factors that, upon activation, translocate from the cytoplasm to the nucleus where they initiate the transcription of target genes (64). In the heart, STAT3 is activated under a variety of stress conditions, such as pressure overload, hypoxia, and acute myocardial infarction. Cardiac STAT3 is phosphorylated in response to hypertrophic stimuli, such as pressure overload (65) and mechanical stress (66). Cardiac myocyte-specific STAT3 mutant mice are more susceptible to cardiac injury after doxorubicin treatment compared to age-matched controls. Furthermore, these mice display an increase in left-ventricular chamber size and heart dysfunction with advancing age (67, 68). STAT3 expression and phosphorylation is severely reduced in failing human hearts (69), which correlates to the development of dilated cardiomyopathy, heart failure, and early death observed in cardiac myocyte-specific STAT3 mutant mice (67, 68). Mice over-expressing STAT3 display myocardial hypertrophy and are protected from doxorubicin-induced apoptosis (67).

STAT3 is also activated by oncoproteins, such as erbB2, and constitutive activation of STAT proteins, in particular STAT3, is observed in transformed cell lines, tumors, and tumor-derived cells (70-72). For example, Fernandes et al (70) showed that STAT3 is constitutively activated in lung epithelial tumor cells and that the inhibition of erbB2 blocks STAT3 activation. ErbB2 and STAT3 co-immunoprecipitate in pancreatic adenocarcinoma cell lines, which also require erbB2 for STAT3 activation (71). The same study also shows that the inhibition of EGFR

had negligible effects on blocking STAT3 phosphorylation, suggesting that erbB2 is required for STAT3 activation in these tumor cell lines.

I sought to test the hypothesis that ErbB2 is required for LIF-induced STAT3 and Erk1/2 activation in adult mouse cardiac myocytes.

**RESULTS**

**ERBB2 IS REQUIRED FOR LIF-INDUCED STAT3 ACTIVATION**

ErbB2 heart-specific mutant and wild type cardiac myocytes were stimulated with serum free media or 1 or 5ng/ml of LIF for five or twenty minutes. Figure 3-1 shows that erbB2 is required for LIF-induced STAT3 activation in adult cardiac myocytes at all concentrations and time points tested.
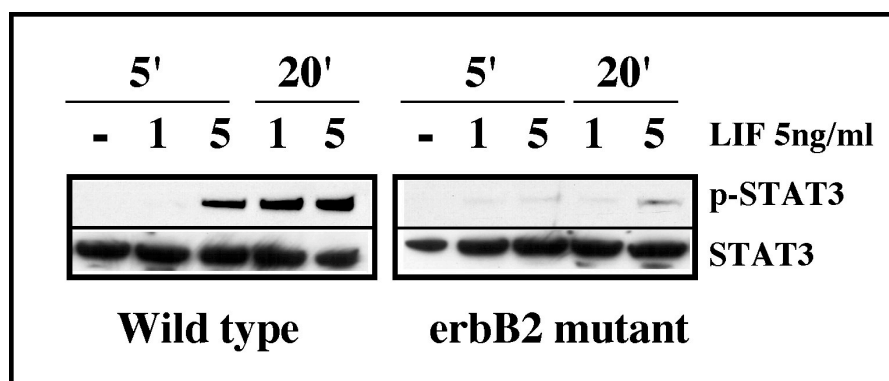
**Figure 3-1 ErbB2 is required for LIF-induced Stat 3 activation in cardiac myocytes**.  Cardiac myocytes isolated from wild type controls and erbB2 heart-specific mutants were serum starved for three hours and stimulated with serum free media or 1 or 5 ng/ml LIF for 5 or 20 minutes.  Cell lysates were subjected to immunoblot analysis using an antibody that detects phosphorylated STAT3 (p-STAT3) and total STAT3 (STAT3) (Cell Signaling).

## ERBB2 IS REQUIRED FOR NRG-INDUCED STAT3 ACTIVATION

In cancer cell lines, NRG stimulation results in the activation of STAT3.  Due to the important role of STAT3 signaling in the heart, we tested whether NRG activates STAT3 in the heart and whether the activation requires erbB2.  In **Figure 3-2**, we show that erbB2 is required for NRG-induced STAT3 activation in adult mouse cardiac myocytes.
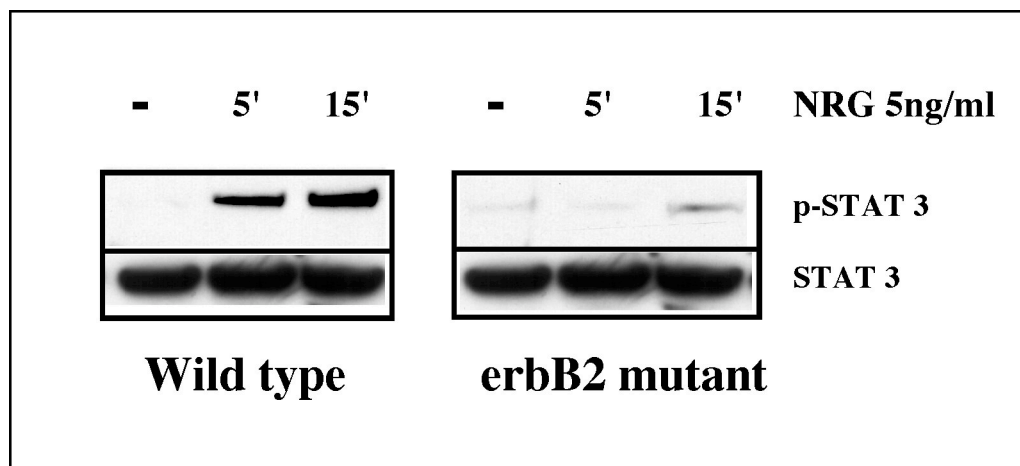
**Figure 3-2   ErbB2 is required for NRG-induced Stat3 activation in cardiac myocytes**. Cardiac myocytes isolated from wild type controls and erbB2 heart-specific mutants were serum starved three hours and stimulated with serum free media or 5 ng/ml of NRG for 5 or 15 minutes.   Cells lysates were harvested and immunoblots were probed with phospho-Stat 3 (top panel) followed by total Stat 3 (lower panel).

**INTERPRETATION OF RESULTS**

It is well established that STAT3 signaling is important for cardiac myocyte survival and hypertrophy and is involved in protecting the heart from injury and heart failure (73). The present study established that erbB2 is required for LIF- and NRG-induced STAT3 activation in adult mouse cardiac myocytes.  Therefore, the loss of STAT3-dependent signaling may play an important role in the dilated cardiomyopathic phenotype observed in the heart-specific erbB2 mutant mice. A

possible mechanism underlying the loss of STAT3 activation in the heart-specific erbB2 mutant mice may be due to the inability of gp130 to bind to erbB2, since it has been established that erbB2 and gp130 form a heterocomplex (15).

The mechanism of NRG-induced STAT3 activation in cardiac myocytes is unknown. In lung cancer cells, NRGs bind to an erbB2/erbB3 heterodimer to initiate STAT3 activation. Due to the absence of erbB3 in wild type myocytes, as well as the inability of NRG to bind to EGFR or erbB2, NRG-induced STAT3 activation in cardiac myocytes may be the result of an erbB2/erbB4/ heterodimer or an erbB2/erbB4/gp130 heterocomplex. It will be of interest to determine whether NRG requires gp130 for STAT3 activation. These data provide new perspectives on the role of NRG-induced STAT3 activation based on the cell-type specific expression of erbB receptors and their associated integrative downstream effects.

## ERBB2 IS REQUIRED FOR LIF-INDUCED ERK1/2 ACTIVATION

In **Figure 3-3**, we show that erbB2 is required for LIF-induced Erk1/2 activation in adult mouse cardiac myocytes.
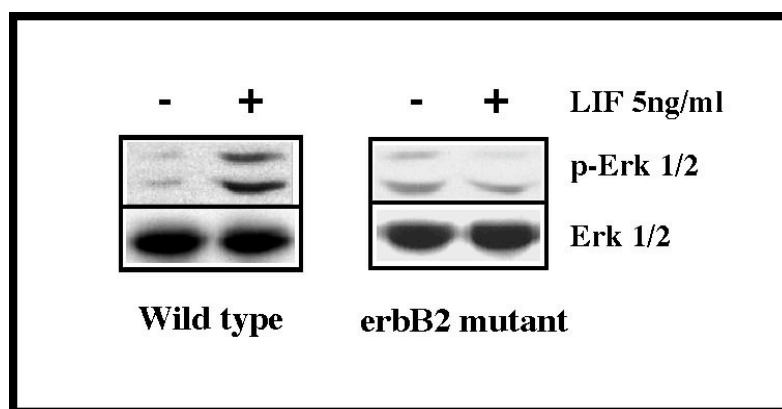
**Figure 3-3 ErbB2 is required for LIF-induced Erk1/2 activation** Wild type and erbB2-deficient adult mouse cardiac myocytes were stimulated with serum- free media or LIF (5 ng/ml) for 10 minutes. Cell lystates were immunoblotted with antibodies for P-Erk1/2 followed by Erk1/2.

## INSULIN SIGNALING IN THE HEART

Additional interactions between NRG and other signaling pathways in the heart, such as insulin-like growth factor-1, have also been described (23). Since insulin stimulation results in Erk1/2 activation in cardiac myocytes (74),we investigated whether erbB2 is required for insulin-induced Erk1/2 activation.

**ERBB2 IS REQUIRED FOR INSULIN-INDUCED ERK1/2 ACTIVATION**

Figure 3-4 shows that erbB2 is required for Insulin-induced Erk1/2 activation in adult mouse cardiac myocytes.
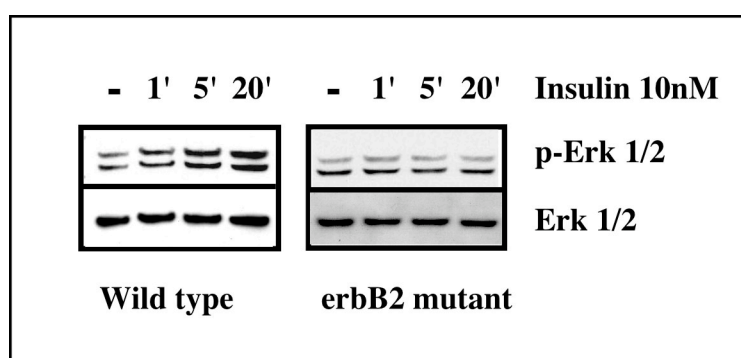


**Figure 3-4 ErbB2is required for Insulin-induced Erk1/2 activation** Wild type and erbB2-deficient adult mouse cardiac myocytes were stimulated with serum-free media or Insulin (10nM) for either 1, 5, or 20 minutes. Cell lystates were immunoblotted with antibodies against p-ERK1/2 and then T-Erk1/2.

**INTERPRETATION OF RESULTS**

The insulin receptor heterotetrameric proteins consist of two polypeptide chains, each with an extracellular α subunit and β subunit. The α subunit contains the ligand binding domain and the β subunit possesses tyrosine kinase activity (75).

Activation of the insulin receptor by insulin results in the phosphorylation of downstream signaling targets, including the insulin receptor substrate- (IRS) 1 and 2, adaptor protein Shc, and the G-protein G$\alpha$q/11 (76). Insulin and contractile activity are key factors regulating glucose utilization in muscle tissue and the amplification of glucose transport is achieved by the redistribution of the glucose transporter 4 (GLUT4) to the plasma membrane (77).

The lipid kinase, phosphoinositide 3-kinase (PI3-K), plays a critical role in glucose uptake by insulin through activation of the serine kinases Akt/protein kinase B (PKB) and protein kinase C (PKC) isoforms (78). PI3-Ks mediate cell survival, proliferation, and motility and are activated in response to growth factor receptor and GPCR stimulation. In contrast to the three type I PI3Ks ($\alpha$, $\beta$, $\delta$) that are activated by tyrosine kinases, PI3K$\gamma$ is activated by the G$\beta\gamma$ subunit of G-proteins and act downstream of GPCRs (78). PI3K$\gamma$ negatively regulates cardiac contractility by reducing cAMP concentration and PI3K$\gamma$ (-/-) mice display enhanced cardiac contractility (79).

Insulin stimulation leads to increased tyrosine phosphorylation of G$\alpha$q/11 and PI3K activation (76). Usui and colleagues (76) found that the tyrosine phosphorylation of G$\alpha$q/11 in response to insulin/insulin receptor signaling participated in glucose transport via cdc42 and PI3K. Furthermore, G$\alpha$q/11 mediated glucose transport stimulation in response to insulin is inhibited by the G protein-coupled receptor kinase (GRK) 2 by disrupting GLUT4 translocation (76). Chronic stimulation by endothelin-

1 leads to desensitization of insulin signaling, decreased tyrosine phosphorlyation of IRS-1 and G$\alpha$q/11, and decreased glucose transport (76).

It will be important to determine how erbB2 modulates insulin signaling. One possibility is that erbB2 is recruited to the activated insulin receptor complex and is responsible for the tyrosine phosphorylation G$\alpha$q/11. The loss of erbB2 would result in the chronic inhibition of G$\alpha$q/11 through its association with GRK2. (see Chapter 4 for more details on G$\alpha$q/11).

Taken together, the data presented in this thesis demonstrates that erbB2 is required for activation of key signaling pathways that play important roles in adult c a r d i a c                                        f u n c t i o n .

# Chapter 4

# General Conclusions and Perspectives

The erbB receptor signaling network has been established as a key regulator and modulator of multiple developmental and physiological processes, including cardiac development and adult cardiac function. The timing, pattern, and tissue distribution of erbB receptors, their ligand affinity, and their ability to form dimers underscores the diversity of their biological responses.

The present dissertation established that the erbB2 tyrosine kinase receptor is required for the integration of multiple cardioprotective signaling pathways (**Schematic 4-1**). We modified the protocol for isolation of adult rat cardiac myocytes for use on adult mouse hearts. The primary adult mouse cardiac myocyte cultures provide a unique model with which to study erbB2-dependent signaling in wild type and heart-specific erbB2 mutant mice that may potentially reveal its role in adult onset cardiomyopathies.
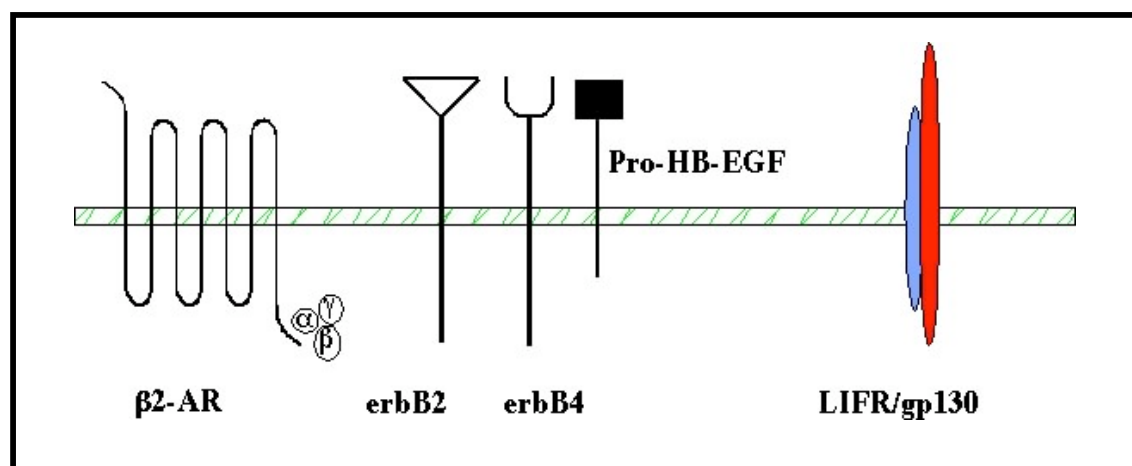


**Schematic 4-1      Functional activation and genetic disruption reveal key signaling pathways in cardiac function.**

G-protein coupled receptors (GPCRs), Receptor tyrosine kinases (RTKs), and IL-6 cytokine receptors signaling pathways are important for multiple developmental and physiological processes. A primary intracellular target of all these receptor families is the Erk1/2 MAPK survival pathway. Therefore, in Chapter 2, we focused our studies on the role of erbB2 in Erk1/2 activation in response to erbB receptor ligands, EGF and NRG, and GPCR agonists, as a way to evaluate the potential cross-talk between these receptors and the convergence of signals through Erk1/2 activation.

Although evidence for the cross-talk between receptor tyrosine kinases and GPCR signaling pathways has been well established, the mechanism of such cross-talk is not well understood. For example, EGFR can be transactivated by GPCR agonists under certain conditions and has been implicated in the activation of MAPK by GPCRs *in vitro* based on studies using pharmacological inhibitors of EGFR (16). However, it is not known whether the RTK-GPCR cross talk occurs *in vivo* and what roles, if any, other erbB members play in such cross talk. My studies revealed that erbB2, not EGFR, is essential for cross talk between RTKs and GPCRs leading to integrated down-stream signal activation in cardiac myocytes.

# ERBB RECEPTOR LIGAND-INDUCED ERK1/2 ACTIVATION IN ADULT MOUSE CARDIAC MYOCYTES

Adult mouse cardiac myocytes express EGFR, erbB2 and erbB4. We first established that erbB2 is required for NRG-induced Erk1/2 activation in isolated adult mouse cardiac myocytes (**Schematic 4-2**). These data support the concept that NRG requires erbB2 for signaling in the heart, since it selectively binds erbB4 and erbB3, the latter of which is not expressed in cardiac myocytes. It is interesting to note that erbB4 is not able to compensate for the loss of erbB2 for maintenance of NRG-dependent signaling, suggesting NRG requires an erbB2/erbB4 heterodimer for signaling.

Conversely, we determined that erbB2 is not required for EGF-induced Erk1/2 activation (**Schematic 4-2**), indicating that the loss of erbB2 does not result in a global loss of Erk1/2 activation. Furthermore, the lack of cardiac side effects in patients treated with EGFR inhibitors *in vivo* correlates to the notion that EGFR is not essential for adult cardiac homeostasis (51). Thus, the genetic deletion of erbB2, the targeted deletion of erbB2 in cardiac myocytes, and the cardiac side effects observed by the functional loss of erbB2 in the heart strongly suggests that erbB2 is critical for multiple aspects of cardiac development and function.
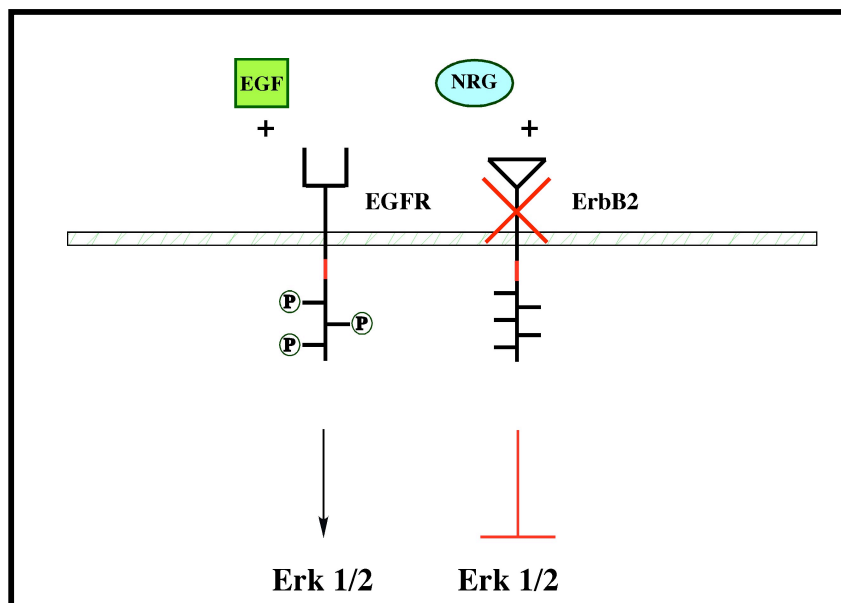
**Schematic 4-2**       **The loss of erbB2 results in the loss of NRG-induced Erk1/2 activation, but not EGF-induced Erk1/2 activation.**

# REQUIREMENT OF ERBB2 FOR GPCR-DEPENDENT ERK1/2 ACTIVATION

The studies shown in this thesis provide key evidence linking erbB2 to GPCR ligand-induced Erk1/2 activation in the presence of functional EGFR in primary cultures of adult cardiac myocytes. The data show for the first time that erbB2 is required for the activation of Erk1/2 in response to isoproterenol (ISO), angiotensin II (ANGII), phenylephrine (PE), urocortin 2 (Ucn 2), glucagons (GLU), and vasoactive intestinal peptide (VIP), in adult cardiac myocytes (**Schematic 4-3 A**). These results verified that the loss of GPCR agonist-induced Erk1/2 activation was not due to a loss

of cell surface expression of their respective receptors: wild type and erbB2-deficient cardiac myocytes were still responsive to GPCR agonist-induced cAMP elevation and $\beta 2$-AR and CRFR2$\beta$ mRNA levels were similar in wild type and erbB2-deficient cardiac myocytes.
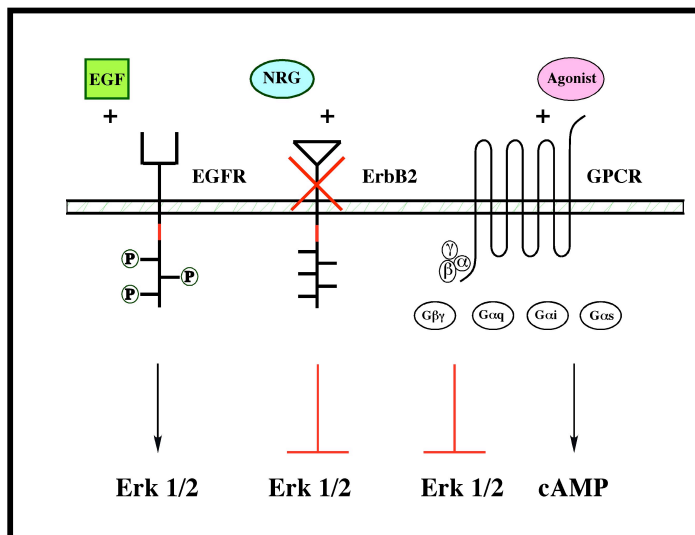


**Schematic 4-3 A     ErbB2 is required for NRG- and GPCR agonist-induced Erk1/2 activation, but not for EGF-induced Erk1/2 activation.**

The novelty of these data is that the loss of GPCR agonist-induced Erk1/2 activation occurs in the presence of functional EGF/EGFR signaling, which is in contrast to previous studies showing that EGFR is the RTK responsible for this response (52). Likewise, Andreev et al (41) found that EGFR was dispensable for Erk1/2 activation in response to GPCR agonist stimulation in EGFR-null mouse

embryonic fibroblasts. To verify that EGFR is not required for GPCR agonist-induced Erk1/2 activation, a cell line lacking endogenous EGFR, B82L cells, was utilized in the present study to show that EGFR is not required for ISO- or Ucn 1/2-induced Erk1/2 activation (**Schematic 4-3 B**).
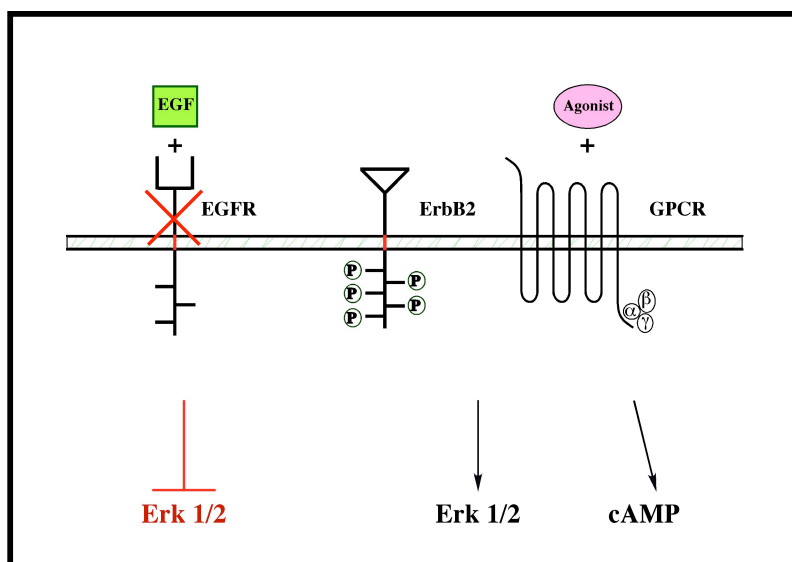


**Schematic 4-3 B**      **EGFR is not required for GPCR agonist-induced Erk1/2 activation.**

## ERBB2 FORMS A COMPLEX WITH AND IS TRANSACTIVATED BY GPCRS *IN VIVO* AND *IN VITRO*

In an effort to establish the mechanism of erbB2-GPCR cross-talk, I conducted studies that show erbB2 forms a ligand-induced complex with β2-AR *in vivo* and *in*

*vitro*.  In addition, erbB2 is transactivated by Ucn 2 in adult cardiac myocytes and forms a ligand-induced complex with CRFR2β *in vitro*.  The sub-cellular localization of these molecules, such as in caveolae and/or transverse t-tubules (2) may facilitate heterocomplex formation and transactivation in response to ligand stimulation.  We also show that the entire erbB2 molecule, in addition to a functional kinase domain, are required for cross-talk with GPCRs.  The ability of erbB2 to complex to multiple classes of receptors suggests a potential mechanism for erbB2 in integrating signaling networks.

**WORKING HYPOTHESES AND FUTURE DIRECTIONS**

As with all conditional knockout and transgenic mouse models, the key question regarding all the phenotypes observed is whether the consequence of the loss of the target protein is due to developmental defects or the direct result of the functional loss of the protein in a given tissue at the desired time point.  For example, the heart-specific erbB2 mutant mice express erbB2 post-natally.  At birth, erbB2-heart specific mutant mice progressively lose expression of erbB2 in cardiac myocytes and develop dilated cardiomyopathy at six weeks after birth.  Whether the post-natal absence of erbB2 alters the expression of key cardioprotective signaling molecules prior to the onset of cardiomyopathy is not know.  It is possible that the absence of erbB2 changes the expression or function of protein tyrosine kinases, phosphatases,

and/or other signaling molecules. However, our ability to reproduce our findings of the loss of GPCR agonist-induced Erk1/2 activation in the absence of erbB2 in multiple cell types suggests it is not due to a developmental lack of erbB2. Acute deletion of the erbB2 floxed allele in cardiac myocytes by treatment with an adeno-associated cre virus is not possible due to the fact that adult mouse cardiac myocytes are not viable beyond a 24 hour period. Therefore, generation of an inducible cre erbB2 floxed line would need to be generated to knockout erbB2 in adult hearts to fully test this hypothesis. As a corollary, the *in vivo* hemodynamic studies could be repeated on wild type mice treated with and an anti-erbB2 antibody or inhibitor. If the pharmacological inhibition of erbB2 in wild type hearts results in a similar phenotype to the heart-specific erbB2 mutants in response to GPCR agonist infusion, this would suggest that the present data is not due to a developmental loss of erbB2 in the heart but rather is due to a functional loss of erbB2 in adult cardiac myocytes.

## IS ERBB2 REQUIRED FOR PRO-HB-EGF CLEAVAGE AND EGFR TRANSACTIVATION IN RESPONSE TO GPCR STIMULATION?

Based on the present data, erbB2 may be required for GPCR agonist-induced Erk1/2 activation by regulating pro-HB-EGF cleavage and transactivation of EGFR. For example, the EGF and NRG family of growth factors, which includes heparin-binding growth factor (HB-EGF), exist as transmembrane precursors requiring

cleavage by cell surface proteases for the production of soluble ligands (80). It is established that the activation of GPCRs results in HB-EGF cleavage by a matrix metalloprotease (MMP) resulting in transactivation of the EGFR and Erk1/2 activation (16). Based on the current data, one possibility is that erbB2 may be required for GPCR-agonist induced MMP cleavage of HB-EGF. The loss of erbB2 would therefore result in a failure to generate soluble HB-EGF for binding to and activation of EGFR and subsequent Erk1/2 activation, perhaps due to loss of clustering of the protease with its substrate (**Schematic 4-3 C**).
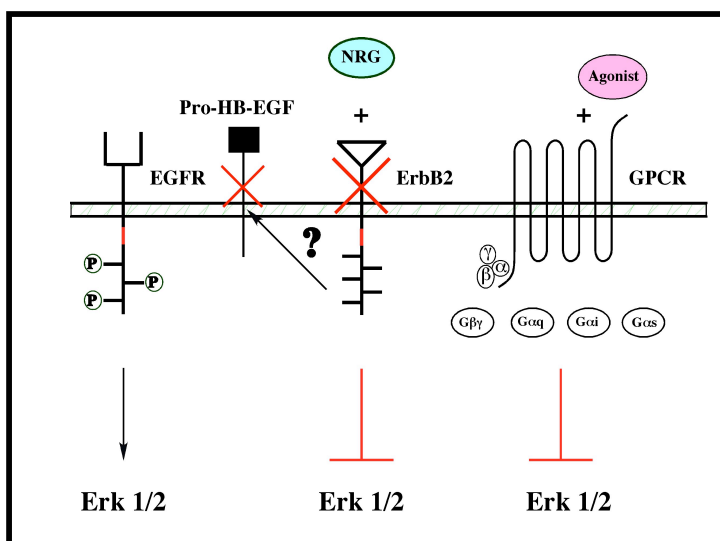


**Schematic 4-3 C**      **Does the loss of erbB2 result in loss of HB-EGF cleavage in response to GPCR agonist stimulation?**

This hypothesis is based on data demonstrating that MMP cleavage of pro-HB-EGF cleavage is dependent upon the activation of Erk1/2 (81). In addition, loss of HRG/HER (human NRG/erbB) receptor signaling in breast cancer cell lines results in the loss of Erk1/2 activation and MMP-9 enzymatic activity (82). Conversely, erbB2-mediated Erk1/2 activation results in the upregulation and increased activity of MMP-9 (82). Thus, the loss of erbB2-mediated Erk1/2 activation in response to GPCR agonists may result in the loss of MMP-dependent pro-HB-EGF cleavage.

It will be important to first determine whether GPCR agonist stimulation results in pro-HB-EGF cleavage in wild type adult mouse cardiac myocytes. This can be determined by measuring the levels of soluble HB-EGF present in the media with or without stimulation by GPCR agonists. If HB-EGF is detected at higher levels post-GPCR agonist stimulation, the same experiments can be conducted in erbB2-deficient cardiac myocytes. The loss of HB-EGF cleavage in the absence of erbB2 in response to GPCR agonist stimulation would suggest that erbB2 is required for pro-HB-EGF cleavage and that erbB2 is acting upstream of MMPs.

However, pro-HB-EGF cleavage likely represents only one way in which GPCRs activate Erk1/2 via erbB2. For example, the data presented in this thesis demonstrates that B82L EGFR-null/erbB2 positive cells are still responsive to GPCR agonist-induced Erk1/2 activation. It is not known whether or not these cells express pro-HB-EGF. If so, soluble HB-EGF would presumably bind to an erbB2/erbB4 heterodimer, since HB-EGF does not bind erbB2. I propose that HB-EGF binds to an

erbB2/erbB4 heterodimer, since erbB2-deficient cardiac myocytes are not responsive to GPCR agonist-induced Erk1/2 activation, thus arguing against an erbB4 homodimer or EGFR/erbB4 heterodimer. If, however, B82L EGFR-null cells do not express pro-HB-EGF, that result would indicate that the cleavage of pro-HB-EGF is not the primary mechanism of GPCR agonist-induced Erk1/2 activation via erbB2. It is likely that erbB2 is required for multiple mechanisms of Erk1/2 activation.

## POTENTIAL ROLE OF Gαq/11 IN GPCR AGONIST-INDUCED ERK1/2 ACTIVATION VIA ERBB2

Another likely hypothesis of how erbB2 may mediate GPCR agonist-induced Erk1/2 activation is via recruitment and activation of Gαq/11. There are four members of the Gq α subunit family: Gαq, Gα11, Gα14, and Gα15/16 (83). Gαq/11 are ubiquitously expressed both in development and in the adult (83). Gα subunits have tyrosine residues near their COOH-termini and can be tyrosine phosphorylated *in vitro* in response to GPCR agonist stimulation (84). Ligand activation of GPCRs coupled to Gαq/11 stimulate the formation of $IP_3$, which results in the release of intracellular $Ca^{2+}$ (85). The formation of $IP_3$ is blocked by pre-treatment of cells with tyrosine kinase inhibitors (84). Umemori and colleagues (86) also showed that protein tyrosine phosphatases play an important role in the activation of Gαq/11. Umemori et al (86) suggest a situation in which Gαq/11, at rest, is not tyrosine phosphorlyated, a process

that is controlled by protein tyrosine phosphatases. Upon ligand binding, non-phosphorylated Gαq/11 proteins are able to interact with their respective GPCR, where GDP is exchanged to GTP. Subsequently, activated receptor tyrosine kinases phosphorylate Gαq/11(Y355), resulting in dissociation of G proteins from the receptor. Tyrosine 355 of Gαq/11 appears to be essential for GPCR-mediated formation of IP$_3$ (84). The tyrosine phosphorylated Gαq/11 is thereby able to activate PLCβ, that, in turn, activates multiple downstream effectors, including PI3K.

Gαq/11 double mutants die at embryonic day 11 and display cardiac abnormalities (83). The hearts of the Gαq/11 double mutants show severe thinning of the myocardial layer, malformed trabeculae, and enlarged hearts filled blood (83). I propose that activated GPCRs recruit erbB2 monomers for initiation of downstream signaling cascades. The GPCR agonist-induced erbB2 homodimer would thus result not only in erbB2 activation but also in tyrosine phosphorylation of Gαq/11, which is recruited to the activated GPCR heterocomplex. Once phosphorylated, both erbB2 and Gαq/11 would activate downstream effectors, such as Erk1/2 and PI3-K.

Whether activated Gαq/11 plays a role in MMP activation is still not known. However, the activation of the angiotensin II type 1 (AT$_1$) receptor, which is primarly coupled to Gαq/11, results in HB-EGF cleavage. The activation of an AT$_1$ mutant receptor lacking the Gq binding site does not result in HB-EGF cleavage (87). Taken together, recruitment of erbB2 monomer(s) to activated GPCRs may involve the

participation of both erbB receptor (Shc, Grb2, Shp2) and GCPR-coupled (G-proteins) intracellular effectors for Erk1/2 activation.

## ERBB2 TRANSACTIVATION OF GPCRS

Several additional key questions are raised by these findings. How does erbB2 recruitment modulates GPCRs?  First, it will be interesting to test whether GPCRs are tyrosine phosphorylated by erbB2 in response to agonist stimulation.  Many GPCRs, such as the β2-AR and CRFR2β, have cytoplasmic tyrosine residues that are known to be important for activation of downstream signaling targets.  For example, tyrosine 350 of the β2-AR potentiates insulin-induced Erk1/2 activation (88).  These phosphorylated tyrosine residues may be important for recruiting intracellular proteins required for the activation of downstream signaling cascades, similar to the mechanism utilized by activated erbB receptors.

Second, is erbB2 is required for GPCR internalization and does the loss of erbB2 alter the kinetics of internalization/desensitization? The same tyrosine residue of β2-AR required for potentiation of insulin-induced Erk1/2 signaling is important for desensitization and targeting of the receptor with clathrin (89).  Whether or not erbB2 plays a role in these processes should be investigated (**Schematic 4-5**).

The ligand independent-activated structure of the erbB2 extracellular domains could explain why multiple receptor signaling networks recruit erbB2. Bypassing the ligand binding process would facilitate rapid signal transduction via recruitment of erbB2 monomers to activated receptor complexes. In addition, the number of erbB2 monomers recruited to a given signaling complex still needs to be explored.
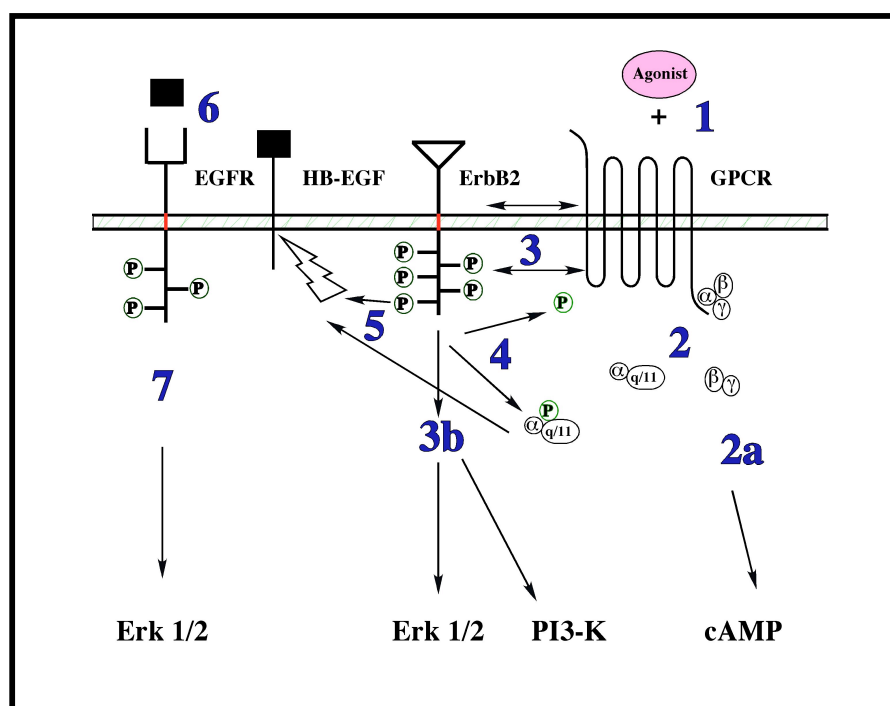


**Schematic 4-5    Potential mechanism of erbB2-mediated signaling in response to GPCR agonists.**

**CONCLUDING REMARKS**

Data presented in this thesis leads to the conclusion that erbB2 signaling in the heart extends beyond erbB receptor signaling networks and is required for signaling induced by multiple classes of ligands in adult mouse cardiac myocytes. The present studies support the concept of an essential, novel, mechanism requiring erbB2 as a co-receptor for GPCR signaling in the heart and expand understanding of the dynamic and intricate relationship between RTKs and GPCRs. The ability of erbB2 to function as a signal integrator in response to a variety of stimuli to coordinate the cross-talk between multiple signaling pathways in the heart may reveal a potential new role for erbB2 function in other tissues.

# Appendix

**CELL SURFACE EXPRESSION OF cDNAs**



| **ErbB2** | **Flag β2-AR** | **Merge** |
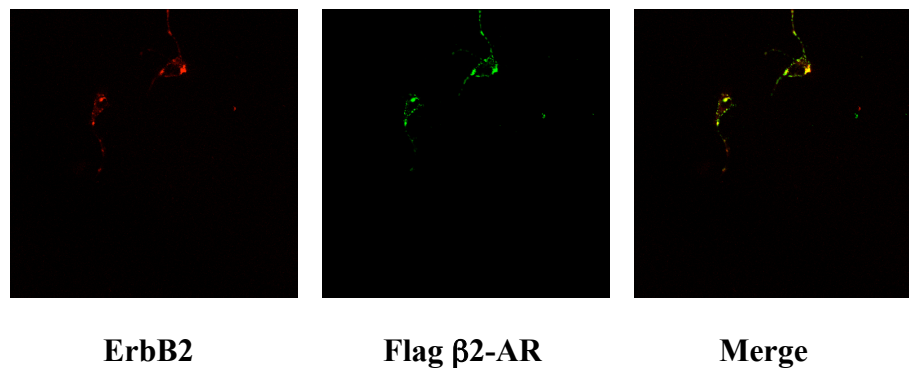
**Figure A-1    Cell surface expression of transfected cDNA constructs**  This image depicts Cos7 cells that were co-transfected with 0.5 μg of erbB2 + 0.5 μg of Flag-β2-AR.  Both erbB2 (red) and Flag-β2-AR (green) are expressed on the cell membrane of the same cells. (see  Immunofluorescence experimental details below)



| **erbB2ΔECD** | **erbB2−KD** | **erbB2ΔICD** |
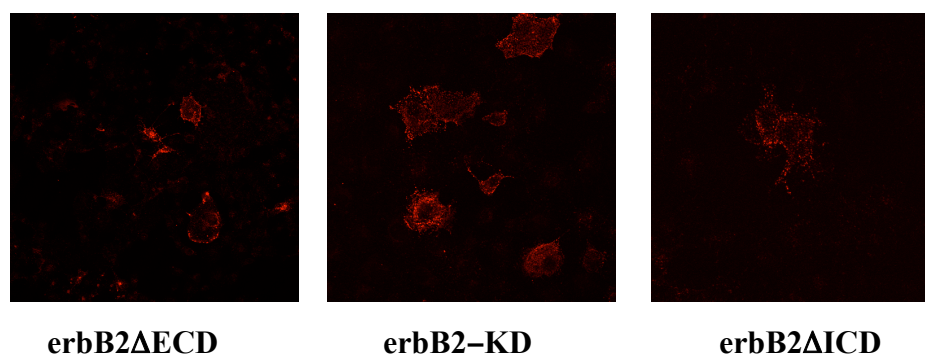
**Figure A-2    Cell surface expression of transfected cDNA constructs** This image depicts Cos7 cells that were co-transfected with 0.5 μg of pcDNA3 + 0.5 μg of erbB2 mutant constructs.  All erbB2 constructs are expressed on the cell membrane. (see Immunofluorescence experimental details below)

# PROTOCOLS AND METHODS

**LIVE IMMUNOFLUORESCENCE PROTOCOL**

This method is used to detect cell surface expression of proteins on live cells using antibodies targeting the extracellular domains of desired proteins.

• Plate COS7 cells on glass cover slips coated with poly-D lysine (1:100) and allow to grow to 60-80% confluency.

• Transfect appropriate amount of cDNA based on the size of the well used (i.e. 0.5 μg/well of 12-well dish). For co-localization studies, the control wells of each cDNA tested should be co-transfected with pcDNA3 vector control.

• Grow cells overnight in 37°C incubator in DMEM supplemented with 10% fetal calf serum and pen/step.

• Wash cells two times with HEPES + 5% fetal bovine serum. Incubate cells with primary antibody (1:500) diluted in HEPES + 5% fetal bovine serum for thirty minutes at room temperature.

• Wash cells three times with HEPES + 5% fetal bovine serum. Incubate cells with corresponding secondary antibody (1:500) diluted in HEPES + 5% fetal bovine

serum for thirty minutes at room temperature.

• Wash cells three times with HEPES + 5% fetal bovine serum. Wash once with 1X PBS.

• Fix cells with 95% ice cold ETOH. Be gentle at this stage because cells may come off cover slip.

• With forceps, pick up cover slip, blot on paper towel, and mount on slide using vectashield.

Antibodies used for immunofluorescence of erbB2 wild type and mutant constructs and β2-AR

**Primary Antibodies**

α-erbB2, α-erbB2-KD, α-erbB2ΔICD        **AB3 mouse monoclonal**

erbB2ΔECD                                **HA rabbit polyclonal**

β2-AR                                    **Flag rabbit polyclonal**

**Secondary Antibodies:**

erbB2, erbB2-KD,erbB2ΔICD        **donkey anti-mouse Cy3 (red) Jackson Labs**

erbB2ΔECD                        **donkey anti-rabbit Cy3 (red) Jackson Labs**

β2-AR                            **goat anti-rabbit FITC (green) Jackson Labs**

**COS7 TRANSIENT TRANSFECTION PROTOCOL**

The following protocol is for use in 6-well tissue culture plates.

•      Grow cells to 70-80% confluency.

•      For six well plates, use one µg of cDNA per well. Prepare a master mix of DMEM + Transfectene (BIORAD) using the following ratio:

2 µl Transfectene (Biorad)/1µg DNA/100 µl DMEM.

•      Aliquot master mix into a new tube specific for each transfection group.  Add cDNA to aliquoted master mix for a final concentration of 1µg/well of a 6-well dish. For co-transfection studies, balance single transfected wells with vector control (such as pcDNA3).

•      Incubate at room temperature for 20-30 minutes.

•      The final volume of each well will be 1ml. Add DMEM + 10% fetal calf serum to each well for a final volume of 1ml/ug cDNA and add to COS7 cells.

•      Incubate cells overnight in 37°C incubator.

•      Rinse the cells with warm serum- free media the next morning and add 2mls of warm DMEM + 10% fetal bovine serum- + pen-strep.

•      Incubate cells overnight in 37°C incubator.

•      For stimulation studies, rinse cells three times with warm serum- free media. Add 1ml serum- free media and starve for 6-8 hours prior to stimulation.

**IMMUNOPRECIPITATION PROTOCOL**

• Rinse the cells twice with ice cold 1xPBS + protease and phosphatase inhibitors (1:500) (Sigma) on ice.

• Add 1ml (for six well dish) of ice cold RIPA buffer + protease and phosphatase inhibitors (1:500) (Sigma) on ice to lyse the cells. Incubate on ice for 20 minutes.

• Harvest cell lysate and place in ice cold 1.5ml eppindorf tube.

• Add 20ul of protein A/G beads and incubate on ice for 20 minutes. Spin at 4°C for 20 minutes at 12K rpm.

• Transfer supernatant into new, ice-cold tube. Take 100ul for determination of protein concentration and for western blot of total protein.

• Use equal amounts of protein for IP. Add antibody (1:000) concentration to each tube. Place on orbitron in cold room and incubate for 4-6 hours.

• Add 20 µl protein A/G beads and incubate for 4 hours on orbitron in cold room.

• Spin down IP lysates for 30-60 seconds at 4°C. Beads will form a pellet. Remove supernatant and wash with Hepes. Spin for 30-60 seconds to pellet beads. Repeat three times.

• After washes, resuspend beads in 40 ul of 2X Urea protein loading dye.

**ADULT MOUSE CARDIAC MYOCYTE CULTURES**

**A BIOLOGICAL MODEL TO ASSESS RECEPTOR SIGNALING ROLES IN CARDIAC FUNCTION.**

**PROTOCOL**

• All buffers and media must be prepared fresh each day of experiment (see recipes below). Warm buffers and media in 37°C water bath.

• Warm the water-jacketed glass perfusion system (condenser) with built-in bubble trap to 37°C. The condenser is connected to a peristaltic perfusion pump. Fill condenser with calcium free Heart Media (Joklik Modified Media). Set flow rate to 2.75 ml/min.

• Fill one 50ml conical tube with ice cold heart media and another with DMEM supplemented with 10%FBS/pen/strep (Complete media).

• Heparinize (250 U/mouse ip) adult mice 5 min prior to being anesthetized with 0.25% (wt/vol) Avertin.

• Spray thoracic region with 70% ETOH and wipe down prior to incision.

• Quickly excise heart and place in ice cold complete media. Gently squeeze heart with blunt forceps to remove blood.

• Transfer heart to new dish containing ice cold heart media. Remove excess

tissue, if necessary. Attach the plastic cannula to a 1ml syringe containing ice cold heart media. Ensure that there are no air bubbles in syringe or cannula! Insert cannula into aorta. Tie aorta to cannula with silk thread. Push 0.2ml media through heart.

• Remove cannula from syringe and hang vertically onto the perfusion apparatus.

• Perfuse heart for 1 min with pre-warmed 37 C Ca2+-free heart media. The heart should visibly swell when being perfused.

• After 1 minute, perfuse heart with digestion buffer containing 0.75 mg/ml collagenase type 2 (Worthington Biochemicals) in 0.1% (wt/vol) BSA for 9–15 min (20 μM CaCl2) in supplemented Joklik's media.

• Remove heart from cannula. Cut off ventricles and mince with sterile razor blades in a dish containing 1ml digestion buffer. Transfer to a conical tube filled with 4mls warm digestion buffer. Digest heart for another 3–6 min in the collagenase solution by gently triturating with sterile pipette.

• Once digested, add 20 mls of warm wash buffer to stop digestion.

• Filter 20 mls through 70 micron nylon mesh in a fresh 50 ml conical tube.

• transfer filtrate into two 15 ml conical tubes and allow cardiac myocytes to pellet. Once a pellet is observed, remove supernatant and add another 10 mls of warm wash buffer. Repeat twice.

• Begin calcium re-introduction step by adding 0.25, 0.5, 0.75, 1, 1.25mM calcium in 5 minute increments to cells in wash buffer to slowly equilibrate the cells to

1.25mM calcium.

• Resuspend final cardiac myocyte pellet in warm, complete media at a density of 1 x $10^3$ cells/well on 10 µg/ml pre-laminated (Sigma) coated plates for 1 h (6 cm$^2$) in 37°C incubator.

• For signaling studies, cells are rinsed with serum-free DMEM and incubated with serum free DMEM for 4 hours. After stimulation, the cells are harvested in ice-cold RIPA buffer with protease and phosphatase inhibitors (1:500) (Sigma). The lysates will be used for total protein analysis for MAPK activity.

• For cAMP assays, media is changed to DMEM/0.1% FBS for a minimum of 2 hours before treatment. The cells are pre-incubated for 90 min in the presence of 0.1 mM 3-isobutyl-1-methylxanthine and then exposed to ligands. Intracellular cAMP is extracted and measured from triplicate wells using an RIA kit (Biomedical Technologies, Stoughton, MA). Forskolin (1 µM) is added to the cells to test their viability and ability to stimulate cAMP. Values for pmol/well cAMP are determined using the Prism 3.0 software (GraphPad Inc., San Diego, CA) from three independent experiments.

• For immunoprecipitation studies, adult mouse cardiac myocytes are resuspended in a final volume of 2 mls of serum-free DMEM. One ml of cells is

treated with serum-free media and the other with ligand. Each ml of cells is pelleted by centrifugation and resuspended in 1 ml of ice cold RIPA buffer containing protease and phosphatase inhibitors (1:500) (Sigma). Cell lysates are pre-cleared by incubation with 20 ul of protein A/G agarose beads (Santa Cruz) on an orbitron for 20 min at 4 °C. Following centrifugation (10,000 rpm, 10 min 4 °C), the cell lysates are incubated with antibody overnight on an orbitron at 4 °C. The following morning, 20 ul of protein A/G beads are added to lysates and incubated for 4 hours on an orbitron at 4 °C . Lysates are centrifuged and pellets are washed three times with ice-cold HEPES and transferred to a clean microcentrifuge tube before addition of 40 $\mu$l of 2X~ Urea protein loading buffer.

## CARDIAC MYOCYTE CULTURE BUFFERS AND MEDIA

Joklik Modified Media

| | |
|---|---|
| NaCl | 112 mM |
| KCl | 5.4 mM |
| $MgCl_2$ | 1mM |
| $NaH_2PO_4$ | 9 mM |
| D-glucose | 11.1 mM |

Add to Joklik (for Heart Media)

| | | |
|---|---|---|
| Hepes | 2380 mg/L | 10mM |
| Taurine | 3750 mg/L | 30mM |
| DL-carnitine | 400 mg/L | 2mM |
| Creatine | 300 mg/L | 2mM |

Adjust pH to 7.4 with NaOH and Filter

Collagenase Solution (Digestion Buffer)

| | |
|---|---|
| Heart Media | 150 mls |
| Collagenase Type II | 0.75 mg/ml |
| BSA | 150 mg (.01%) |
| $CaCl_2$ | 20 μM (30ul of 100mM stock) |

Wash Solution

| | |
|---|---|
| Heart Media | 100 mls |
| BSA | 1000 mg (1%) |
| $CaCl_2$ | 20 μM (20ul of 100 mM stock) |

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