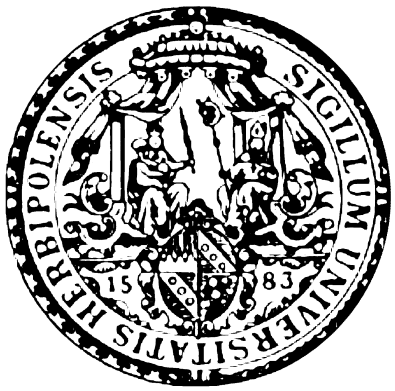


# β-adrenergic receptors and ERK1/2-mediated cardiac hypertrophy

# β-adrenerge Rezeptoren und ERK1/2-vermittelte Herzhypertrophie

Doctoral thesis for a doctoral degree
at the Graduate School of Life Science,
Julius-Maximilians-Universität Würzburg
Section Biomedicine

Submitted by
Marie Vidal

From
Dijon, France

Würzburg, 2013

# SUPERVISORY COMMITTEE

| | |
|---|---|
| Primary supervisor | Professor Dr. Martin Lohse |
| Second supervisor | Professor Dr. Kristina Lorenz |
| Third supervisor | Professor Dr. Anne-Marie Lompré |
| Chairperson | Professor Dr. Manfred Gessler |

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# Affidavit

I hereby confirm that my thesis entitled beta-adrenergic receptors and Erk1/2-mediated cardiac hypertrophy is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

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# ABSTRACT

β-adrenergic receptors (β-ARs) participate strongly in the development of cardiac hypertrophy and human heart failure. Stimulation of β-adrenergic receptors with catecholamines as well as cardiac overexpression of $\beta_1$-ARs or of $G\alpha_s$-proteins in transgenic mice induces cardiac hypertrophy. However, direct activation of their downstream targets, such as adenylyl cyclase (AC) or protein kinase A do not promote a significant degree of cardiac hypertrophy. These findings suggest that additional events may occur and that these events require $G\alpha_s$-protein activation. A hypertrophic pathway involving $G\alpha_q$-protein coupled receptors has recently been described. Upon activation of $G\alpha_q$-coupled receptors $G\beta\gamma$-subunits are released from $G\alpha_q$ and bind directly to the activated Raf/Mek/Erk cascade. Direct interaction between βγ-subunits and activated Erk1/2 leads to an additional autophosphorylation of Erk2 at threonine 188, which mediates cardiac hypertrophy. Murine hearts, as well as isolated cardiomyocytes present an increase in $Erk2^{Thr188}$-phosphorylation upon β-AR activation. Similarly overexpression of phosphorylation deficient Erk2 mutants ($Erk2^{T188S}$ and $Erk2^{T188A}$) reduces β-AR mediated cardiomyocyte hypertrophy. Increase in left ventricular wall thickness, fibrosis and up-regulation of natriuretic peptide synthesis, which are physiological features for cardiac hypertrophy, are strongly inhibited in transgenic mice with a cardiac expression of $Erk2^{T188S}$ after two weeks of sustained isoproterenol treatment. It could further be shown in this work that β-AR mediated cardiac hypertrophy requires two distinct pathways initiated by $G_s$-protein activation: the canonical phosphorylation of Erk1/2 via adenylyl cyclase and the direct interaction of released βγ-subunits with activated Erk1/2. Coincidence of both events leads to $Erk2^{Thr188}$-phosphorylation, which activates then different transcription factors responsible for cardiac hypertrophy. Sequestration of βγ-subunits by overexpression of the C-terminus of GRK2 βark-ct and inhibition of adenylyl cyclase efficiently reduced the hypertrophic response to isoproterenol, whereas direct activation of AC by forskolin failed to induce $Erk2^{Thr188}$-phosphorylation and cardiomyocyte hypertrophy. These findings may help to develop new therapeutic strategies for the prevention of cardiac hypertrophy and maladaptive remodeling of the heart.

# ZUSAMMENFASSUNG

Chronische Aktivierung von β-Adrenorezeptoren (β-ARs) durch Katecholamine ist ein Stimulus für kardiale Hypertrophie und Herzinsuffizienz. Ebenso führt die Expression von $\beta_1$-ARs oder $G\alpha_s$-Proteinen in genetisch modifizierten Mäusen zu Hypertrophie und Herzinsuffizienz. Allerdings führt die direkte Aktivierung dem $G\alpha_s$ nachgeschalteten Komponenten des β-adrenergen Signalwegs wie z.B. die Aktivierung der Adenylylcyclase (AC) oder der Proteinkinase A (PKA) nicht im signifikanten Ausmaß zur Herzhypertrophie. Diese Ergebnisse deuten darauf hin, dass zusätzlich zu dem klassischen Signalweg, auch weitere durch $G\alpha_s$-Proteine aktivierte Komponenten in die β-adrenerg vermittelte Hypertrophieentwicklung involviert sind. Interessanterweise wurde vor kurzem ein hypertropher Signalweg beschrieben, der eine direkte Involvierung von Gβγ-Untereinheiten bei der Induktion von Herzhypertrophie durch die extrazellulär-regulierten Kinasen 1 und 2 (ERK1/2) zeigt: Nach Aktivierung $G\alpha_q$-gekoppelter Rezeptoren binden Gβγ-Untereinheiten an die aktivierte Raf/Mek/Erk Kaskade. Die Bindung der freigesetzten Gβγ-Untereinheiten an Erk1/2 führt zu einer Autophosphorylierung von Erk1/2 an Threonin 188 (bzw. Thr208 in Erk1; im folgenden $Erk^{Thr188}$-Phosphorylierung genannt), welche für die Vermittlung kardialer Hypertrophie verantwortlich ist. In dieser Arbeit konnte nun gezeigt werden, dass auch die Aktivierung von β-ARs in Mäusen sowie von isolierten Kardiomyozyten zur Induktion von $Erk^{Thr188}$-Phosphorylierung führt. Darüberhinaus führte die Überexpression von Erk2 Mutanten ($Erk2^{T188S}$ und $Erk2^{T188A}$), die nicht an Threonin 188 phosphoryliert werden können, zu einer deutlich reduzierten Hypertrophieantwort von Kardiomyozyten auf Isoproterenol. Auch die kardiale Expression der $Erk2^{T188S}$ Mutante im Mäusen verminderte die Hypertrophieantwort auf eine 2-wöchige Isoproterenol-Behandlung deutlich: Die linksventrikuläre Wanddicke, aber auch interstitielle Fibrose und Herzinsuffizienzmarker wie z.B. BNP waren signifikant reduziert. Weiterhin konnte in dieser Arbeit gezeigt werden, dass tatsächlich ein Zusammenspiel von Gα und Gβγ-vermittelten Signalen zur Induktion von $Erk^{Thr188}$-Phosphorylierung und damit zur Induktion von β-adrenerg vermittelter Hypertrophie notwendig ist. Während die Hemmung von Gβγ-Signalen mit dem C-Terminus der GRK2 oder die Hemmung von Adenylylzyklase eine $Erk^{Thr188}$-Phosphorylierung und eine

Hypertrophieantwort nach Isoprenalingabe effektiv reduzierten, führt die alleinige Aktivierung von Adenylylzyklase nicht zu einer Hypertrophieantwort. Diese Ergebnisse könnten bei der Entwicklung neuer möglicher therapeutischen Strategien zur Therapie β-adrenerg induzierter Herzhypertrophie und Herzinsuffizienz helfen.

# INTRODUCTION

Heart failure is the major cause of mortality in the Western World, and thus generates a great human and economic problem for the modern society. Indeed a third of the patients die within a year of diagnosis and no efficient treatment has been developed so far. People are diagnosed with heart failure when their hearts are no more able to pump blood properly to cover the systemic demands. Symptoms such as fatigue, insomnia, anxiety, depression, shortness of breath, edema dizziness and nausea reduce the quality of life of heart failure patients (Bernardo et al, 2010; Heineke & Molkentin, 2006). Heart failure can be induced by several stimuli, such as longstanding hypertension, congenital malformation or myocardial infarction. One of the major risk factors for myocardial infarction, arrhythmia and sudden death is cardiac hypertrophy (Heineke & Molkentin, 2006). Over the last decades, the understanding of cellular and molecular mechanisms involved in the regulation of cardiac hypertrophy has therefore been an important challenge.

## I. CARDIAC HYPERTROPHY

### 1. The cardiac muscle

The mammalian heart is a muscular pump, which regulates the circulation of the blood and thus, the distribution of oxygen and nutrients in the whole organism. For this reason, it is the first organ to form and to develop in the embryo and cardiac abnormalities often trigger fetal death (Olson, 2004; Woodcock & Matkovich, 2005).

The cardiac muscle, also called myocardium, is covered by the endocardium (at the inner layer) and the epicardium, which protects the heart from external injuries. Contractile cardiomyocytes, which constitute only 30% of the total cardiac-cell number but represent 80% of the heart's mass are surrounded by an extracellular matrix and non-myocyte cells, such as fibroblasts, vascular smooth muscle cells, endothelial cells and mast cells (Bernardo et al, 2010). In mammals, cardiomyocytes are established as terminally differentiated muscle cells and are

responsible of the cardiac contractile force. Indeed, at birth or few days after, these cells are no more able to divide and are thus not able to participate in the regeneration of injured myocardium (Anversa & Nadal-Ginard, 2002; Bernardo et al, 2010; Woodcock & Matkovich, 2005). Heart growth from childhood to adulthood results then from cardiomyocyte growth and not from cell proliferation (Woodcock & Matkovich, 2005).

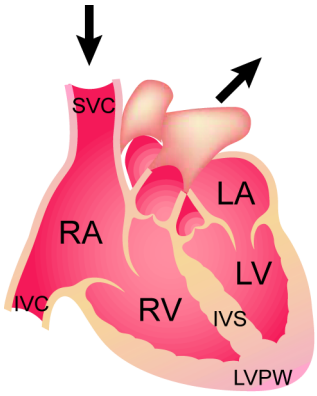



**Figure 1. Illustration of a normal heart.** RA, right atria; RV, right ventricle; LA, left atria; LV, left ventricle; IVS, interventricular septum; LVPW, left ventricular posterior wall; IVC inferior vena cave. Modified from Towbin & Bowles, 2002.

As in any muscle cell, sarcomeres are the elementary contractile component of a cardiomyocyte. Z-disks make the junction between two sarcomeres, leading to their synchronized contraction and relaxation. Bunches of sarcomeres form myofilaments, fundamental component of myofibrils. And cardiomyocytes are structured by bundles of myofibrils. The synchronized contraction and relaxation of any individual cardiomyocyte ensures enough pressure to eject the blood in the arteries and efficient cardiac activity.

## 2. Cardiac hypertrophy

### a. Concentric and eccentric cardiac hypertrophy

Under increased workload the heart adapts through hypertrophic remodeling. Individual cardiomyocytes enlarge their size and increase their protein synthesis. Cardiac hypertrophy is, thus, characterized as an increase in cardiac mass. Depending on the initiating stimuli different changes in shape of the heart are initiated. Eccentric hypertrophy is the common type of hypertrophy that is caused

by volume overload. Typically, cardiac mass is increased due to an enlargement of the left ventricular chamber, cardiomyocyte length as well as septum and left ventricle wall thickness. In contrast, concentric hypertrophy is initiated by pressure overload. This type of hypertrophy is characterized by an increase in left ventricular wall thickness and cardiomyocytes enlarged in width. Ventricular dilation is absent in concentric hypertrophy (Figure 2) (Heineke & Molkentin, 2006).

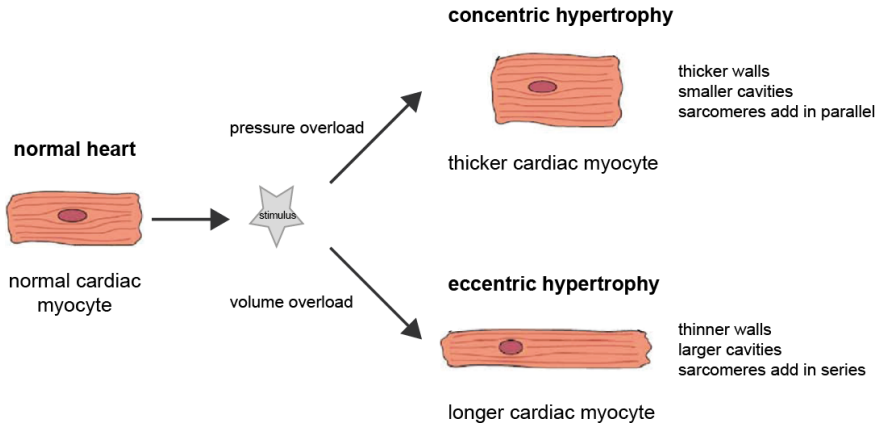



**Figure 2. Concentric *versus* eccentric cardiac hypertrophy.**

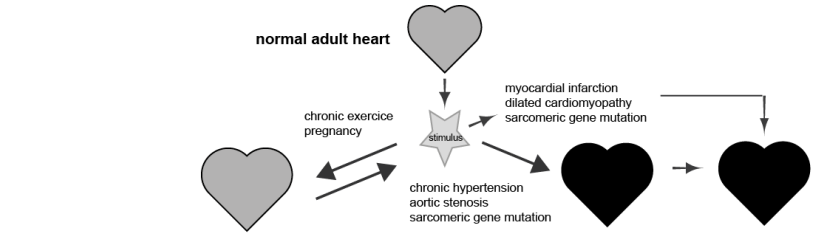
### b. Physiological and pathological cardiac hypertrophy

Initially, cardiac hypertrophy augments cardiac pump function and reduces ventricular wall tension. However in the long term, cardiac contractility can decrease and can be followed by left ventricular dilation, loss of contractile function and heart failure. Nevertheless, there are physiological types of hypertrophy such as postnatal growth, cardiac growth during pregnancy and exercise-induced hypertrophy. They are reversible and characterized by normal cardiac structure and function. In contrast, pathological hypertrophy is induced by sustained pressure or volume overload in a disease setting, myocardial infarction or ischemia associated with coronary artery disease or abnormalities that contribute to cardiomyopathy such as genetic mutations or diabetes (Figure 3).

In both physiological and pathological hypertrophies, actin synthesis is up-regulated and sarcomeres reorganize, inducing enlargement of cardiomyocytes either into eccentric or concentric shape. In comparison to pathological hypertrophy, physiological hypertrophy is not associated with fibrosis.

Fibrillar collagen network guarantees structural cohesion of adjacent cardiomyocytes and therefore facilitates proper ventricular pump function. Cell death, i.e. apoptosis or necrosis, is increased in pathological hypertrophy. Since myocytes are unable to regenerate, loss of cells is repaired by excessive accumulation of collagen, which is also called interstitial fibrosis. Fibrosis stiffens the ventricles and impairs contraction and relaxation. It also deteriorates electrical coupling of cardiomyocytes. Further, oxygen diffusion is disturbed and thereby provokes myocardial ischemia (Bernardo et al, 2010).

On molecular level, in pathological hypertrophy the expression patterns several proteins such as of contractile proteins ($\alpha$- and $\beta$-myosin heavy chain (MHC)), fetal genes (atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), $\alpha$-skeletal actin) and calcium-handling proteins. Moreover, the metabolism of hypertrophied hearts is disturbed: Fatty acid oxidation decreases and glucose metabolism increases (Figure 3).




| biological features | physiological hypertrophy | pathological hypertrophy | cardiac dilation heart failure |
|---|---|---|---|
| cardiac function | normal | depressed | advanced cardiac disfunction |
| heart size | proportionnal chamber enlargement | disproportional chamber enlargement | disproportional chamber enlargement |
| cardiac fibrosis | no fibrosis | fibrosis | extensive fibrosis |
| fetal gene expression | normal | upregulation of ANF, BNP… | upregulation of ANF, BNP… |

**Figure 3. Physiological *versus* pathophysiological cardiac hypertrophy.**

## 3. **Hypertrophic stimuli**

Cardiac hypertrophy is initiated by cellular events that first take place at the cell membrane. These events include mechanical stretch as well as paracrine and autocrine humoral factors such as catecholamines, endothelin 1, angiotensin II or

insulin-like growth factor 1 (IGF1). Direct interaction of hormones with specific membrane-bound receptors activates intracellular signaling cascades leading to cardiac hypertrophy. Hypertrophic signaling pathways are diverse and complex and crosstalk with each other. However, some factors seem preferentially be involved in physiological or pathological hypertrophy. For instance IGF1 levels in the heart increase after exercise whereas elevated Angiotensin II or catecholamine levels are typically associated with pressure overload and heart failure. The understanding of the differences in underlying pathways of physiological and pathological hypertrophy is a logical approach for the development of new therapeutic.

A wide range of intracellular pathways has been described so far, such as the calcineurin-NFAT circuit. Upon calcium activation calcineurin dephosphorylates nuclear factor of activated T-cells (NFAT), which translocate to the nucleus and participate in hypertrophic gene expression. Protein kinases, PKC or PKD, are also prohypertrophic regulators in response to pressure overload. Other signaling pathways involve small G proteins, HDACs that regulate chromatin remodeling or mitogen activated protein kinases.

# II. G PROTEIN COUPLED RECEPTORS

## 1. G protein coupled receptors

### a. Definition

G protein coupled receptors (GPCRs) constitute the largest and ubiquitously expressed class of plasma membrane receptors. They consist of three extra- and three intracellular loops, with an extracellular N-domain and an intracellular C-tail (Palczewski et al, 2000; Rasmussen et al, 2007; Warne et al, 2008). Light, taste, odors, neurotransmitters, hormones, and different chemical classes, such as calcium ions, peptides, amino acids, nucleotides, lipids and fatty acid derivatives act as agonist on these receptors. Because of their diverse biological functions, GPCRs play a major role as target in drug development (Rockman et al, 2002).

Especially to be emphasized is the discovery of specific β-adrenergic receptor antagonists by Sir James Black, which now represent the major therapeutics in the treatment of hypertension and heart failure (Baker et al, 2011).

### b. GPCR activation

Direct binding of agonists to the active site induces conformational changes within the transmembrane domain, which leads to the activation of heterotrimeric guanine-nucleotide-binding proteins called G proteins. G proteins consist of a $\alpha$-subunit and a $\beta/\gamma$-subunit complex. Four subfamilies of G proteins based on $\alpha$-subunit similarities have been described. They include $G_s$ (stimulatory G protein), $G_i$ (inhibitory G protein), $G_q$ or $G_{11/12}$ and lead to activation or inhibition of different effector proteins. While activation of $G\alpha_s$ or $G\alpha_i$ initiates or inhibits adenylyl cyclase (AC) respectively, stimulation of $G\alpha_q$ triggers phospholipase C activation (PLC) (Figure 4). Activity of $G\alpha$ subunits is based on their ability to exchange guanine diphosphate (GDP - inactive state) to guanine triphosphate (GTP - active state). Stimulated G proteins dissociate from the activated receptor and propagate extracellular signals into the cell via activation of their respective downstream effector proteins. In turn, these effectors translate the signal and generate second messengers, which induce cellular responses through different intracellular signaling pathways. In parallel, dissociated $G\beta\gamma$-subunits target also their own signaling pathways, such as mitogen activated protein kinases (MAPKs), phosphatidylinositol-3-kinase (PI3K), PLC$\beta$, AC, the small GTP-binding proteins (Clapham & Neer, 1997; Hendriks-Balk et al, 2008; Patel et al, 2009; Schmitt & Stork, 2002; Wettschureck & Offermanns, 2005). Although GPCRs are known to couple preferentially to one specific G protein, some of them, such as muscarinic receptors or $\beta_2$-adrenergic receptors have been shown to signal through different G proteins.

### c. GPCR desensitization

Receptor desensitization or receptor downregulation terminates G protein activation by GPCRs. Activated GPCRs are phosphorylated by G protein coupled receptor kinases (GRKs), which leads to $\beta$-arrestin recruitment. Direct interaction of $\beta$-arrestin with the receptor not only prevents any further GPCR/G protein coupling but also targets the receptor to clathrin-coated pits for endocytic internalization resulting either in receptor recycling to the plasma membrane or in receptor degradation (Figure 4) (Hendriks-Balk et al, 2008; Rockman et al, 2002). GPCRs can also be downregulated by second messenger-dependent kinases such as protein kinase A (PKA) or C (PKC). In comparison to GRKs, PKA can phosphorylate both stimulated and unstimulated GPCRs (Hendriks-Balk et al,

2008). Gβγ-subunits also participate in GPCR desensitization and downregulation by recruiting the receptor kinases GRK2 and GRK3 (Clapham & Neer, 1997).

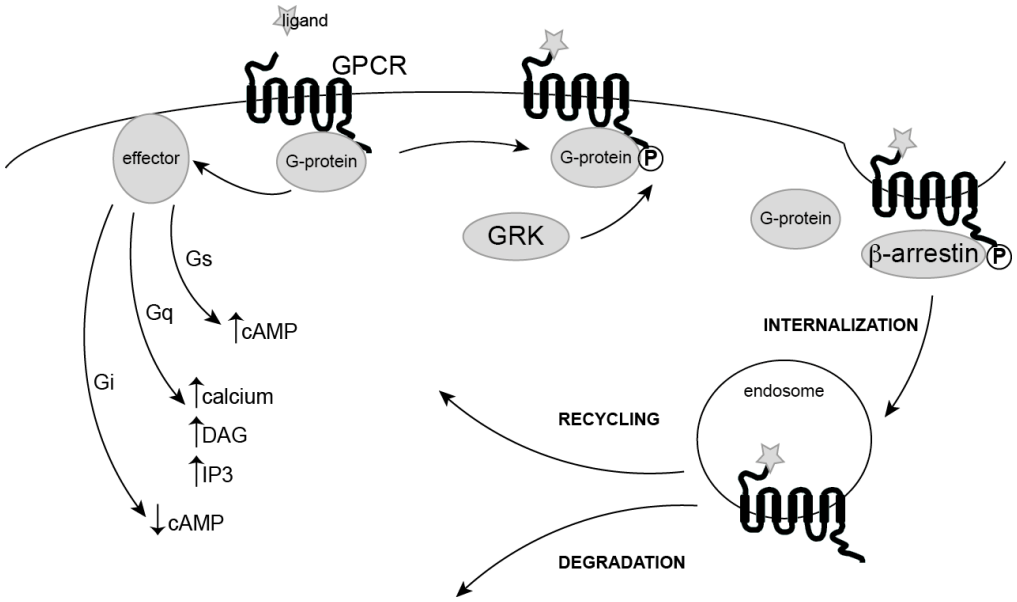



**Figure 4. GPCR signaling.** Ligand stimulated GPCRs promote intracellular signaling through specific G proteins (Gs, Gq or Gi). Activated receptors are inactivated by G protein coupled receptor kinase (GRKs) mediated phosphorylation and subsequent binding of β-arrestin. β-arrestin prevents the binding between the receptor and the G protein and mediates the internalization of GPCRs in endosomes. Internalized receptors are either recycled to the plasma membrane or degraded.

## 2. β-adrenergic receptors

β-adrenergic receptors (β-ARs) play an important role in the development of cardiac hypertrophy. These receptors are activated by catecholamines, such as noradrenaline or adrenaline that are secreted by the sympathetic nervous system. In the heart three subtypes of β-ARs have been described. They regulate a wide range of biological processes, which include heart pacemaker activity, myocardial contractility, and vascular smooth muscle tone. In addition to their cardiac activity they also regulate glucose and lipid metabolism (Lohse et al, 2003; Zheng et al, 2005). $\beta_1$-ARs are the most prominent receptors with a ratio of ~80:20 of $\beta_1$-ARs and $\beta_2$-ARs in a healthy human heart. In addition, a minor fraction of $\beta_3$-ARs is

expressed in the myocardium and modulates myocyte functions (Lohse et al, 2003; Xiao et al, 1999; Barki-Harrington et al, 2004).

### a. β-adrenergic receptor signaling

Although $\beta_1$-ARs and $\beta_2$-ARs are highly homologous, they are not redundant and promote different subcellular mechanisms. While $\beta_1$-adrenergic receptors mediate their signaling pathway only through stimulatory G proteins ($G_s$), $\beta_2$-ARs couple first to $G_s$ but upon PKA mediated phosphorylation they can switch to inhibitory G proteins ($G_i$). $\beta_3$-ARs couple to $G_i$-proteins and a nitric oxide synthase pathway and may, therefore, to some extent counteract the $\beta_1$-AR-mediated cardiac contractility (Barki-Harrington et al, 2004; Gauthier et al, 1998; Lohse et al, 2003).

β-ARs modulate the activity of adenylyl cyclase (AC), which generates the second messenger cyclic adenosine 3',5' monophosphate (Campbell et al, 1995). In cardiomyocytes, cAMP activates cAMP dependent protein kinase A (PKA), which phosphorylates several proteins involved in the excitation-contraction coupling and energy metabolism including phospholamban, L-type calcium channels, troponin I, ryanodine receptors, myosin binding proteins and protein phosphatase inhibitor-1 (Dzimiri, 2002; Feldman et al, 2005; Lohse et al, 2003; Metrich et al, 2008).

Activation of L-type calcium channels and ryanodine receptors induce an elevation of intracellular calcium concentration. The cardiac excitation-contraction coupling is essentially controlled by cytosolic calcium in cardiomyocytes since $Ca^{2+}$ ions are direct activators of myofilaments. Extracellular calcium enters the cells via activated L-types $Ca^{2+}$ channels, which in turn triggers the release of calcium from the sarcoplasmic reticulum (Heximer et al, 1999) via ryanodine receptors (RyRs). Thereby the intracellular $Ca^{2+}$ concentration increases approximately ten-fold. Free $Ca^{2+}$ diffuses then through the cytosol and binds to troponin allowing direct interaction of actin and myosin filaments and, thus, cardiomyocyte contraction (Figure 5). For myocyte relaxation, calcium is removed from the cytosol by its re-uptake into the SR via sarco/endoplasmic reticulum $Ca^{2+}$ ATPase (SERCA) or transported out of the cell involving the sarcolemmal $Na^{+}/Ca^{2+}$ exchanger and the $Ca^{2+}$ ATPase. Thereby $Ca^{2+}$ levels return to diastolic levels, allowing $Ca^{2+}$ to dissociate from troponin and, thus, separating actin from

myosin filaments (Bers, 2002; Endoh, 2008; Lipskaia et al, 2009; Lompre et al, 2010; Woodcock & Matkovich, 2005).

In addition to the PKA-dependent manner, excitation-contraction coupling and calcium handling are regulated by other cAMP effectors: the exchange proteins directly activated by cyclic AMP (Epac). Although Epac are less known than PKA, they can initiate RyR phosphorylation via the $Ca^{2+/}$calmodulin-dependent protein kinase II (CaMKII) and, thus, modulate intracellular $Ca^{2+}$ (Lompré et al, 2010; Métrich et al, 2010).

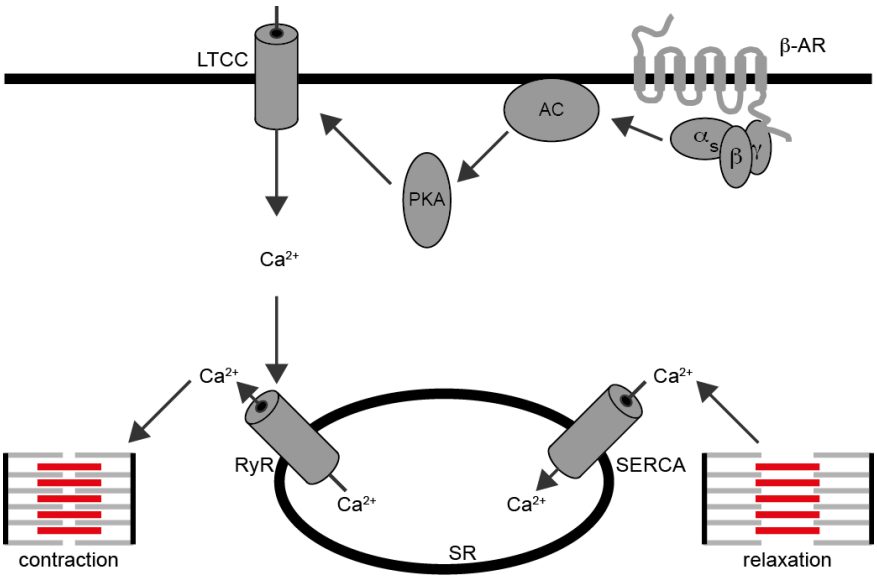



**Figure 5. contraction/relaxation cycle.** Upon activation, β-adrenergic receptors (β-AR) activate adenylyl cylclase via Gas proteins. In turn AC activate protein kinase A (PKA), which phosphorylates L-type calcium channels (LTCC). Cardiac contraction is initiated by calcium influx through activated (LTCC) after. This increase of cellular calcium concentration triggers activation of ryanodine receptors (RyR) and calcium release from the sarcoplasmic reticulum. Free calcium associates with troponin C and stimulates contraction. Actin (black) and myosin (red) filaments slide over one another. Dissociation of calcium from troponin C causes relaxation and its reuptake into the sarcoplasmic reticulum via SERCA.

Via activation of stimulatory G proteins and AC, $\beta_1$-ARs play a dominant role for the enhancement of cardiac chronotropy and ionotropy. In a lesser extend, also $\beta_2$-ARs participate in cardiomyocyte contraction via $G\alpha_s$ and AC stimulation.

However their phosphorylation by PKA induces a shift in G protein coupling towards $G\alpha_i$ proteins, which attenuates the positive inotropic effects of $\beta_2$-ARs (Hendriks-Balk et al, 2008; Salazar et al, 2007).

### b. β-adrenergic receptors and cardiac hypertrophy

Clinical studies with heart failure patients revealed an up-regulation of the sympathetic system and elevated levels of catecholamines. While an increase in adrenergic system initially improves cardiac contractility, sustained activation of adrenergic receptors is detrimental. It has been shown that pronounced activation of the adrenergic system directly correlates with shortened survival. This is coherent with findings in transgenic mice with chronic activation of β-ARs (Bernardo et al, 2010; Feldman et al, 2005; Lohse et al, 2003; Osadchii, 2007; Salazar et al, 2007). In transgenic mice, cardiac overexpression of $\beta_1$-adrenergic receptors promotes cardiac hypertrophy, interstitial fibrosis and progressive deterioration of cardiac function (Bernardo et al, 2010; Engelhardt et al, 1999; Frey & Olson, 2003). Similar results were obtained in transgenic mice overexpressing $G\alpha_s$ upon chronic catecholamine stimulation (Iwase et al, 1997). In contrast, cardiac overexpression of adenylyl cyclase type 6 (AC6), one of the two major AC isoforms expressed in the heart (AC6 and AC5), failed to promote cardiac hypertrophy. Transgenic mice overexpressing AC6 heart function had elevated levels of cAMP but did not develop hypertrophy and their survival was similar to control mice (Roth et al, 2002). Disruption of AC5 improves left ventricular function and protects against myocyte apoptosis after chronic catecholamine stress (Okumura et al, 2007). Mice with cardiac overexpression of the catalytic domain of PKA showed no difference in heart size compared to control mice. Even after 10 weeks and more they could detect none or only a minor increase in heart-to-body-weight ratios and in the wall thickness. Transgenic mice did not suffer from hypertrophy but developed dilated cardiomyopathy (Antos et al, 2001).

These different observations suggest that β-AR mediated cardiac hypertrophy depends on $G\alpha_s$ protein activation but that direct activation of downstream targets of $G\alpha_s$ is not sufficient. Therefore, other downstream proteins than the canonical signaling pathway seem to be involved in the hypertrophic signaling of βARs. Among the different candidates, extracellular regulated kinases 1/2 seem to be interesting candidates because of their implication in cardiac hypertrophy (Muslin, 2008; Rose et al, 2010).

# III. EXTRACELLULAR REGULATED KINASES

## 1. Definition

The extracellular signal-regulated kinases 1 and 2 (Erk1/2) are members of the mitogen-activated protein kinase (MAPK) family (Avruch, 2007; Seger & Krebs, 1995). Other subfamilies of the MAPKs include c-Jun N-terminal kinase (JNK), p38 MAP Kinases and Erk5 (Kehat & Molkentin, 2010; May & Hill, 2008; Ramos, 2008; Rose et al, 2010; Seger & Krebs, 1995; Shaul & Seger, 2007).

Erk1/2 are ubiquitously expressed and activate about 100 putative targets within the cell. They regulate several intracellular signaling events such as cell cycle progression, proliferation, differentiation, migration, survival, cytokinesis, transcription, apoptosis, senescence, GAP junction formation, actin and microtubule network and cell adhesion and are, thus, involved in many biological processes (Kehat & Molkentin, 2010; Rose et al, 2010; Seger & Krebs, 1995; Shaul & Seger, 2007). Erk1/2 are involved in the regulation of the immune system, in heart development, antigen activation and memory formation. They also respond to various different hormones, growth factors and insulin (Kehat & Molkentin, 2010; Rose et al, 2010; Shaul & Seger, 2007). Since Erk1/2 are involved in so many physiological and biological functions, aberrations in their signaling cascade correlate with diverse pathologies including cancers, diabetes, viral infection and cardiovascular diseases.

Erk1 (44 kDa) and Erk2 (42 kDa) share more than 80% amino acid identity (Chen et al, 2001; Rose et al, 2010). Even though they mostly regulate the same signaling activities and are usually referred as Erk1/2, gene knockout experiments have shown that they are not completely functionally redundant. For example, Erk1 knockout mice have a normal phenotype, while the genetic deletion of Erk2 causes an embryonically lethal genotype (Muslin, 2008; Ramos, 2008; Rose et al, 2010).

## 2. Erk1/2 activation

The core of the MAPK cascades consists of MAPK kinase kinase (MAP3K), MAPK kinase (MAPKK) and MAPK. Each kinase phosphorylates and thus activates the corresponding downstream kinase. Subsequent activation of the MAPK then phosphorylates specific targets that are required for the biological response of the MAPKs (Muslin, 2008; Ramos, 2008; Rose et al, 2010; Shaul & Seger, 2007).

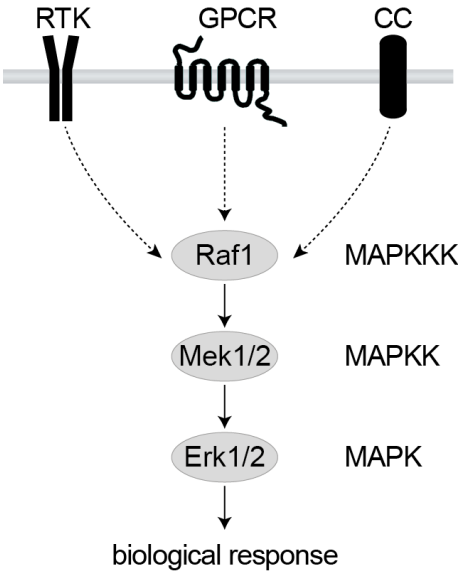



**Figure 6. Scheme of canonical Erk1/2 activation.** The Erk1/2 cascade is induced by receptor tyrosine kinase (RTK), G protein coupled receptors (GPCR) or calcium channels (CC). Erk1/2, which are mitogen-activated protein kinases (MAPK) are prototypically activated by three-tiered sequential phosphorylation events.

Activation of the Erk1/2 cascade is usually initiated by activation of the small G protein Ras. Direct binding of extracellular growth factors (EGF) to their respective transmembrane receptor tyrosine kinases (RTK) activates Ras, which in turn recruits and initiates the activation of the MAP3K Raf-1 at the plasma membrane (Wellbrock et al, 2004). Raf-1 then activates Mek1/2 (mitogen-activated protein kinase/Erk kinase 1/2) by phosphorylation of two serine residues located in the activation loop of Mek1/2 (Ser218 and Ser222 in Mek1). Activated Mek phosphorylates Erk1/2 by phosphorylation of the threonine and tyrosine residues of the conserved Thr-Glu-Tyr ($T^{183}$-E-$Y^{185}$ or TEY) motif in

the phosphorylation loop (Payne et al, 1991). Upon activation, Erk1/2 phosphorylate several transcription factors localized either in the cytosol or in the nucleus, including Elk1, c-Fos, p53, c-Myc, GATA4 or ribosomal S6 kinase (RSKs) (Muslin, 2008; Ramos, 2008; Rose et al, 2010; Shaul & Seger, 2007). Depending on their localization Erk1/2 initiate specific effects. For instance, Elk1, which is localized in the nucleus, enhances transcription of growth related proteins. Nuclear accumulation of Erk1/2 therefore leads to Elk1 phosphorylation, which in turn mediates cell hypertrophy or cell proliferation. In comparison, cytosolic targets such as RSKs promote cell survival (Mebratu & Tesfaigzi, 2009).

The three-dimensional structure of Erk2 has been solved for both the active and the inactive state. Erk2 consists of a smaller N-terminal domain and a larger C-terminal domain connected via a flexible linker region, which allows conformational changes. The domains can either rotate apart to release substrates or together to block the active site. Upon phosphorylation of the TEY motif, the activation loop forms a pocket where substrates bind to the kinase (Chen et al, 2001; Rubinfeld & Seger, 2005). Erk phosphorylates substrates where serine/threonine residues are neighbors to proline (S/T-P). Two docking domains that mediate Erk binding to diverse substrates have been identified: a specific DEF-domain, which is characterized by an S/T-P phosphorylation site adjacent to the sequence Phe-x-Phe-Pro (FxFP) and the common binding site for several MAPKs, the D-domain. Some substrates include only one of these binding domains whereas others are composed of both. For instance, the transcription factor c-Fos contains only the DEF-domain while the transcription factor Elk1 includes both docking sites. Since those two docking sites are bound independently to different regions of Erk1/2, the DEF-domain to a region adjacent to the phosphorylation loop and the D-domain to the common docking site, they direct Erk1/2 to phosphorylate and, thus, activate specific residues. Those sequences are not only found in substrates of Erk1/2 but also in other Erk1/2 interacting molecules such as Mek1/2, which includes the D-domain (Fantz et al, 2001; Ramos, 2008).

Erk downregulation is mainly mediated by dephosphorylation of one or both residues of the TEY motif. This process can be mediated by protein Ser/Thr phosphatases (PPs), protein Tyr phosphatases (PTPs) or dual-specificity MAPK phosphatases (MKPs). Direct interaction between Erk1/2 and PTPs contributes to dephosphorylate the Tyr residues. Although this initial dephosphorylation is

sufficient to inactivate completely Erk1/2, it is usually followed by the removal of the phosphate from the Thr residues by PPs. MKPs remove simultaneously both phosphates from the TEY-motif (Rose et al, 2010; Shaul & Seger, 2007). In addition to these mechanisms, Erk1/2 participate in several feedback loops that decrease or increase their activity. Erk1/2 can inhibit Mek1/2 by phosphorylation of Mek at Thr292 and Thr212, leading to Erk1/2 downregulation. An example of positive feedback loop involves the phosphorylation of the dual phosphatase MKP3. This phosphatase usually dephosphorylates Erk1/2. Phosphorylation of MKP3 by Erk1/2 targets MKP3 degradation and, thus, in turn prevents Erk1/2 inhibition (Ramos, 2008).

The specificity of Erk1/2 signaling is also influenced by the strength, duration and subcellular localization of the Erk signal. In addition, interactions with scaffold proteins contribute to Erk1/2 regulation. The major role of scaffold proteins is to recruit two or more proteins involved in a specific signaling event and to facilitate their interaction. They also bring stability to the cascade components, target them to specific substrates, protect them from phosphatases and prevent crosstalk among similar pathways. As an example, the kinase suppressor of Ras (KSR) forms a complex with Raf, Mek and Erk1/2 and thereby facilitates Erk1/2 phosphorylation and activation. Similarly direct interaction of β-arrestin with the MAPK cascade prevents Erk1/2 translocation to the nucleus resulting in preferential phosphorylation of cytoplasmic substrates (Ramos, 2008; Shaul & Seger, 2007; Tohgo et al, 2002).
Finally non-phosphatase inhibitors such as the Raf kinase inhibitor protein (RKIP) and Sprouty also regulate Erk1/2 signaling cascade. RKIP is a negative regulator of Erk1/2 since it prevents Erk1/2 activity by inhibiting Raf. Phosphorylation of RKIP by PKC inactivates RKIP as Erk inhibitor and thus permits Raf/Mek/Erk activation. In mammalian cells Sprouty4 interacts with B-Raf and sequesters it, which impedes Erk1/2 activation (Lorenz et al, 2003; Ramos, 2008).

## 3. **Erk1/2 and cardiac hypertrophy**

Many studies *in vitro* and *in vivo* have shown that Raf/Mek/Erk cascade plays an important role in mediating cardiac hypertrophy (Muslin, 2008; Rose et al, 2010). Transgenic mice with a cardiac overexpression of either a constitutively active Ras mutant (H-Ras-V12) (Hunter et al, 1995; Zheng et al, 2004) or a

constitutively active Mek1 mutant (Bueno et al, 2000) develop cardiac hypertrophy. An increase in Raf/Mek/Erk activity was also identified in patients exhibiting hypertrophic cardiomyopathies (Rose et al, 2010). Recently Kehat et al. revealed that the Mek/Erk cascade regulates the balance between eccentric and concentric hypertrophy (Kehat et al, 2011).

A novel Erk1/2 autophosphorylation site at residue threonine 188 of Erk2 (Thr208 in Erk1) has recently been identified and this autophosphorylation has been shown to induce cardiac hypertrophy *in vitro* and *in vivo* (Lorenz et al, 2009a; Lorenz et al, 2009b). Thr188-phosphorylation occurs when canonically activated Raf/Mek/Erk cascade binds βγ-subunits of $G_q$-proteins. Gβγ/ERK binding requires the entire activated MAPK cascade and the dimerization of Erk (Figure 7). $Erk^{Thr188}$-phosphorylation occurs in response to hypertrophic stimuli, such as angiotensin, but also to pressure overload induced by transversal aortic constriction (Lorenz et al, 2009a; Lorenz et al, 2009b).

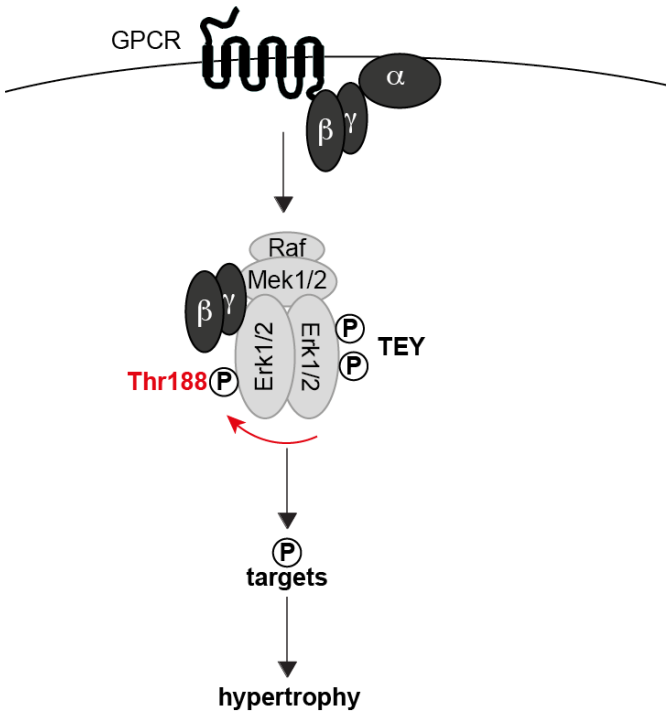



**Figure 7. Erk1/2 mediate cardiac hypertrophy via $Erk^{Thr188}$-phosphorylation.** Association of the Gβγ-subunits released from activated $G_q$-proteins with all the members of the activated Erk cascade induces a third phosphorylation of Erk2 at threonine 188. This new autophosphorylation mediates the hypertrophic response of Erk1/2.

# AIM OF THE THESIS

Sustained activation of β-adrenergic receptors (β-ARs) leads to cardiac hypertrophy, a major risk factor for the development of human heart failure, coronary heart disease and stroke (Barki-Harrington et al, 2004; Frey & Olson, 2003; Lohse et al, 2003; Osadchii, 2007). However the mechanisms involved in the development of cardiac hypertrophy in response to β-ARs are poorly understood. β-ARs belong to the G protein coupled receptor family and signal mainly through the stimulatory $G_s$-protein ($G\alpha_s$). Activation of β-ARs with catecholamine promotes $G\alpha_s$ signaling pathway leading to adenylyl cyclase (AC) activation, which in turn activates protein kinase A. Although this canonical pathway is nowadays well accepted, several lines of evidence suggest that β-adrenergically-induced hypertrophic signaling is not straightforward. While mice with cardiac overexpression of $\beta_1$-adrenergic receptors or $G\alpha_s$-protein develop cardiac hypertrophy (Engelhardt et al, 1999; Iwase et al, 1997; Lohse et al, 2003), in mice with cardiac overexpression of protein further downstream in the β-AR signaling cascade as AC or PKA the development of cardiac hypertrophy is hardly initiated (Antos et al, 2001; Frey & Olson, 2003; Roth et al, 2002). These findings raise the hypothesis that additional events may occur at the level of $G\alpha_s$.

A new hypertrophic pathway involving the βγ-subunits of $G\alpha_q$-proteins and the extracellular regulated kinases (Erk1/2) has recently been described (Lorenz et al, 2009a; Lorenz et al, 2009b). Upon angiotensin activation, Gβγ-subunits are released from activated $G_q$-proteins and bind to the activated Raf/Mek/Erk cascade. Direct interaction of βγ-subunits with the MAPKinase cascade induces a third autophosphorylation of Erk1/2 at threonine 188. Thr188-phosphorylated Erk accumulates in the nucleus and phosphorylates different transcription factors involved in cardiac hypertrophy (Lorenz et al, 2009a; Lorenz et al, 2009b).

Since β-adrenergic receptors activate Erk1/2 (Bogoyevitch et al, 1996; Goldsmith & Dhanasekaran, 2007; Heineke & Molkentin, 2006; Zheng et al, 2010), the aim of the thesis was to investigate whether $Erk2^{Thr188}$-phosphorylation is required for β-ARs mediated cardiac hypertrophy

# MATERIAL

## I. CHEMICALS

| | |
|---|---|
| Acrylamid 30% | Roth |
| Agar | Applichem |
| Agarose | Applichem |
| Albumin (fraction V) | Applichem |
| Ammonium persulfate (APS) | Sigma |
| Ampicillin | Applichem |
| Ascorbic acid | Roth |
| Benzamidine | Sigma |
| 5-bromo-2-deoxyuridine (BrdU) | Sigma |
| Bromophenol blue | Applichem |
| $CaCl_2$ | Applichem |
| Carbachol | Sigma |
| Casein | Applichem |
| Chloroquine | Sigma |
| Citric acid | Merck |
| Collagenase II | Worthington |
| p-Cumaric acid | Fluka |
| Deoxyribonuclease (DNase) | Sigma |
| Dextrose (± D-Glucose) | Applichem |
| 3,3'-Diaminobenzidine (DAB) | Sigma |
| 4',6'-diamidino-2-phénylindole (DAPI) | Invitrogen |
| Diethylaminoethyl-Dextran (DEAE-Dextran) | Pharmasia |
| Diethyl pyrocarbonate | Sigma |
| Dimethyl sulfoxide (DMSO) | Applichem |
| Dulbecco's modified eagle medium (4.5 g/l glucose) | PAN Biotech GmBH |
| Ethylenediaminetetraacetic acid (EDTA) | Merck |
| Ethylene glycol tetraacetic acid (EGTA) | Merck |
| Eosin | Merck |
| Ethanol | Pharmacy |
| Ethidium bromide | Applichem |
| Fetal Calf Serum (FCS) | Biochrom AC |

| | |
|---|---|
| Forskolin | Tocris |
| Glycerol | Pharmacy |
| Glycine | Applichem |
| Hematoxylin | Sigma |
| 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) | Applichem |
| $H_2O_2$ (30% v/v) | Applichem |
| [$^3$H]-isoleucine | Amersham |
| Isoproterenol | Tocris |
| Isoproterenol | Sigma |
| Kanamycin | Applichem |
| KCl | Applichem |
| $KH_2PO_4$ | Merck |
| KH7 | Sigma |
| Laemmli buffer (Roti®-load1) | Roth |
| L-glutamine | PAN Biotech GmBH |
| Luminol | Alexis |
| minimum essential medium | Sigma |
| β-mercaptoethanol | Sigma |
| Methanol | Sigma |
| $MgCl_2$ | Applichem |
| $MgSO_4$ x $7H_2O$ | Merck |
| Milk powder | Applichem |
| NaCl | Applichem |
| NaF | Sigma |
| $Na_2HPO_4$ | Applichem |
| $NaH_2PO_4$ | Applichem |
| $NaHCO_3$ | Applichem |
| $NaN_3$ | Merck |
| NaOH | Applichem |
| $Na_4P_2O_7$ | Sigma-Aldrich |
| $Na_3VO_4$ | Sigma |
| N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) | Sigma |
| NP40 | Sigma |
| Paraformaldehyde (PFA) | Merck |
| PD98059 | Calbiochem |
| Penicillin/Streptomycin | PAN Biotech GmBH |
| *Pertussis toxin* (PTX) | Sigma-Aldrich |

| | |
|---|---|
| Phalloidin (Alexa Fluor® 488) | Molecular Probe |
| Phenylephrine | Sigma |
| Phenylmethylsulfonyl fluoride (PMSF) | Sigma |
| Phosphate buffered saline (PBS) | PAN Biotech GmBH |
| Polylysine | Sigma |
| Protein A Sepharose™ 4 Fast Flow | GE Healthcare |
| PeqGOLD Protein-Marker III | Peqlab |
| Sirius red (direct Red 80) | Aldrich |
| Sodium acetate | Merck |
| Sodium dodecyl sulfate (SDS) | Applichem |
| Sodium glycerophosphat | Fluka |
| Temed | Sigma |
| Trichloroacetic acid TCA | Applichem |
| Tris | Applichem |
| Tris-HCl | Applichem |
| Triton X-100 | Sigma |
| Trypan blue solution (0.4%) | Sigma |
| Trypsin | BD |
| Tween 20 | Sigma |
| Vitamin B12 | Sigma |
| Yeast | Applichem |

## II. CELL LINES

| | |
|---|---|
| Human embryonic kidney 293 cells (HEK293) | (Graham et al, 1977) |
| Monkey kidney cells (COS-7) | (Gluzman, 1981) |

## III. BACTERIA

| | |
|---|---|
| *E. coli* (XL-1 blue) | (Yang, 1992) |
| *E. coli* (DH10) | (Grant et al, 1990) |

# IV. MICE AND RATS

For transgenic mice generation, wild-type Erk2 (T188T) and Erk2$^{T188S}$ (T188S) constructs, which express T188T or T188S under the control of the mouse *$\alpha$-myosin heavy chain ($\alpha$-Mhc*), were injected in fertilized oocytes derived from FVB/N mice (Buitrago et al, 2005; Lorenz et al, 2009a).
For cardiomyocyte isolation pregnant Sprague-Dawley rats (E13 or E14) were purchased from Janvier.

# V. KITS

| | |
|---|---|
| Effecten | Invitrogen |
| ECL Plus Western Blotting Detection system | GE Healthcare |
| Gateway® systems | Invitrogen |
| Pierce® BCA Protein Assay Kit | Thermo Scientific |
| Qiagen Plasmid Plus Maxi Kit | Qiagen |
| Qiagen Plasmid Plus Midi Kit | Qiagen |
| QIAquick® gel extraction kit | Qiagen |
| RNeasy® Kit | Qiagen |
| SsoFast™ EvaGreen® supermix | BioRad |
| Vectastain elite ABC kit | Vector laboratories |

# VI. PLASMIDS

## 1. In pcDNA3 vector (Invitrogen)

From Kristina Lorenz (Lorenz et al, 2009a):
N-terminal tagged HA-murine Erk2 (HA-T188T)
N-terminal tagged Flag-murine Erk2 (Flag-T188T)
N-terminal tagged Flag-murine Erk2$^{T188S}$ (Flag-T188S)
N-terminal tagged Flag-murine Erk2$^{T188A}$ (Flag-T188A)
N-terminal tagged YFP-murine Erk2 (YFP-T188T)
N-terminal tagged YFP-murine Erk2$^{T188A}$ (YFP-T188A)

From Martin Lohse

β1 human adrenergic receptor (Frielle et al, 1987)

β2 human adrenergic receptor (Caron et al, 1988)

From Tanja Eichmann (Eichmann et al, 2003)

βark-ct

### 2. In pAD-CMV-V5-Dest vector (Invitrogen)

From Nadine Yurdagül-Hemmrich

N-terminal tagged Flag-murine Erk2

N-terminal tagged Flag-murine $Erk2^{T188S}$

N-terminal tagged Flag-murine $Erk2^{T188A}$

N-terminal tagged YFP-murine Erk2

N-terminal tagged YFP-murine $Erk2^{T188A}$

From Thomas Wieland (Snabaitis et al, 2005)

YFP-tagged RGS-2

## VII. PRIMERS

### 1. For genotyping (Invitrogen)

| | |
|---|---|
| RF | 5'-aca tag acc tct gac aga ga-3' |
| VF | 5'-ttc cag aag cct cgc aat gtc aa-3' |
| VB | 5'-ccc cct tac cgg tat cag cag aa-3' |
| EB | 5'-gca act cga act ttg ttg ag-3' |

### 2. For RT-PCR (biomers)

| | |
|---|---|
| GAPDH forward | 5'-cat tat cgg cct tga ctg tg-3' |
| GAPDH backward | 5'-tgg caa agt gga cat tgt tg-3' |
| ANF forward | 5'-aca gat ctg atg gat ttc aag aac ctg c-3' |
| ANF backward | 5'-agt gcg gcc cct gct tcc tca-3' |
| Collagenase 3a forward | 5'-aaa cag caa att cac tta cac-3' |
| Collagenase 3a backward | 5'-acc ccc aat gtc ata gg-3' |

### 3. For adenoviruses (Invitrogen)

Flag-Erk2 forward
5'-gggg aca agt ttg tac aaa aaa gca ggc tat gga cta caa gga cga cga cga caa ggc ggc ggc ggc ggc ggc ggg ccc gga gat g-3'
YFP-Erk2 forward
5'-gggg aca agt ttg tac aaa aaa gca ggc tat ggt gag caa ggg cga gga gct gtt c-3'
Erk2 backward
5'-gggg ac cac ttt gta caa gaa agc tgg gtc taa gat ctg tat cct ggc tgg aa-3'

## VIII. MATERIAL AND MACHINES

| | |
|---|---|
| β-scintillation counter | |
| BioRad S1000 Thermal Cycler | BioRad |
| C1000 thermal cycler CFX384™ real-Time System | BioRad |
| Fuchs-Rosenthal cell-counter | Marienfield |
| Fuji medical X-Ray film | Fujifilm |
| Leica DM4000B microscope | Leica |
| Leica RM 2165microscope slices | Leica |
| Leica TCS SP5 confocal microscope | Leica |
| MicroPulse™ | BioRad. |
| NanoDrop 2000c spectrophotometer | Peqlab |
| Osmotic mini-pump (Alzet, 1002) | Durect corporation |
| Transfer-Blot® SD Semi-Dry electrophoretic transfer cell | BioRad |
| Ultra-turrax | IKA labortechnik |
| Vevo2100 high resolution imaging system | VisualSonics |

## IX. ANTIBODIES

### 1. Used for immunoblots

| | |
|---|---|
| Mouse anti-Elk1 | Cell Signaling (9182) |
| Rabbit anti-Erk2 | Santa Cruz (sc-153) |
| Mouse anti-Flag | Sigma (F3165) |
| Mouse anti-HA | Roche (12CA5) |

| | |
|---|---|
| Rabbit anti-Gβ | Santa Cruz (sc-378) |
| Mouse anti-phospho-Elk1 (Ser383) | Cell Signaling (9186) |
| Rabbit anti-phospho-Erk1/2 (TEY) | Cell Signaling (9101) |
| Rabbit anti-phospho-Erk1/2 (Thr188) | (Lorenz et al, 2009a) |

### 2. **Used for immunoprecipitation**

| | |
|---|---|
| Rabbit anti-Erk1/2 | Cell Signaling (9102) |
| Mouse anti-Flag | Sigma (F3165) |
| Mouse anti-HA | Roche (12CA5) |

### 3. **Used for immunohistochemistry**

| | |
|---|---|
| Goat anti-phospho-Elk1 (Ser383) | Imgenex (90121-1) |
| anti-α-actinin (Sarkomer), monoclonal (EA-53) | Sigma (A7811) |

## X. SOFTWARE

| | |
|---|---|
| Diskus (version MIL 8.0b) | Leica |
| Excel (version 14.1.4) | Microsoft |
| Illustrator (version 15.0.0) | Adobe |
| ImageJ (version 1.0) | NIH |
| Leica LAS AF software | Leica |
| Photoshop (version 12.0 x64) | Adobe |
| Prism (version 4.0a) | GraphPad |
| Cardiac measurement Software | VisualSonics |

# METHODS

## I. CELL CULTURE

### 1. Cell culture

Human embryonic kidney 293 cells (HEK293) (Ambrosio & Lohse, 2012; Graham et al, 1977) and monkey kidney cells (COS-7) (Gluzman, 1981; Lorenz et al, 2009a) were cultured in DMEM (4.5g/l glucose) supplemented with 10% (v/v) fetal calf serum (FCS), penicillin (100U/l), streptomycin (100µg/ml) and L-glutamine (2mM) and incubated at 37°C and 7% $CO_2$. Every three or four days, cells were split 1:3 to 1:10.

### 2. Isolation of neonatal rat cardiomyocytes

Neonatal rat cardiomyocytes (NRCMs) were isolated by enzymatic dissociation of the ventricles of 1-to-2-day-old Sprague Dawley rats. Collected ventricles were cut in small pieces and digested either with trypsin or collagenase, as described below.

#### a. Trypsin digestion

Hearts were collected on ice in sterile CBFHH buffer supplemented with penicillin (100U/ml) and streptomycin (100µg/ml). After removal of atria, the ventricles were transferred in tryspin solution (7.5ml) and cut in small pieces. Every 5 minutes the supernatant with dissociated cells was collected in a 50ml tube containing fetal bovine serum (7.5ml) to stop enzymatic activity and to prevent further digestion of collected cells. Fresh trypsin solution (7.5ml) was added to minced ventricles. After complete digestion of ventricles, collected cell suspensions were centrifuged for 10 minutes (700rcf; room temperature). The supernatant was then discarded and pellets were resuspended in 30ml minimum essential medium (MEM; Sigma; M1018) supplemented with 5% (v/v) FCS, penicillin (100U/ml), streptomycin (100µg/ml) and L-glutamine (2mM). The solution was then filtered through a cell mesh (40µm) to separate cell and tissue debris and freshly isolated cells. Cells were incubated for one hour at 37°C by

1% $CO_2$ to isolate cardiomyocytes from fibroblasts and other non-myocardial cells present in ventricles, such as vascular smooth muscle cells, endothelial cells and mast cells (Bernardo et al, 2010). Non-myocyte cells attach faster to the culture dish than cardiomyocytes, which remain in solution in the medium (Blondel et al, 1971; Chlopcikova et al, 2001; Simpson & Savion, 1982). After 1h of incubation, called “pre-plating”, cardiomyocytes could be separated from fibroblasts.

**b. Collagenase digestion**

As described above, hearts were collected on ice in sterile 1*Ads buffer. Ventricles were transferred to collagenase solution (8ml), cut in small pieces and incubated in a shaking water bath for 20 minutes for tissue digestion. The supernatant with the dissociated cells was then collected in 2ml fetal bovine serum. The cell suspension was centrifuged for 5 minutes (800rpm; room temperature) and the cell pellet was resuspended in FCS (4ml). This procedure was repeated 5 times. The collected cell-suspension was then centrifuged for 5 minutes (800rpm; room temperature). Supernatant was discarded and pellets were resuspended in 25ml minimum MEM supplemented with 5% (v/v) FCS, penicillin (100U/ml), streptomycin (100µg/ml) and L-glutamine (2mM). The solution was then filtered through a cell mesh (40µm) and freshly isolated cells were pre-plated for one hour at 37°C by 1% $CO_2$.

**c. Cell counting and splitting**

Non-myocyte cells attached faster to the plate surface whereas cardiomyocytes mainly remain in suspension in the medium (Blondel et al, 1971; Chlopcikova et al, 2001; Simpson & Savion, 1982). After one hour of pre-platting cardiomyocytes were separated from non-myocyte cells. The medium was therefore carefully collected in a sterile beaker. And each plate was washed once with MEM supplemented with 5% (v/v) FCS, 100µM 5-bromo-2-deoxyuridine (BrdU), penicillin (100U/ml), streptomycin (100µg/ml) and L-glutamine (2mM) to collect remaining cardiomyocytes.

50µl of the cell-suspension were then mixed with 50µl of trypan blue solution (0.4%; Sigma; T8154). Trypan blue solution stains dead cardiomyocytes in blue whereas living cells remained uncolored. Living and dead cells could therefore be differentiated under a microscope. In a Fuchs-Rosenthal cells-counter living cells were counted. NRCMs were then plated at the respective densities on

cell-culture dishes coated with poly-lysine as indicated below. For NRCM culture, MEM was supplemented with 5% (v/v) FCS, 100µM BrdU, penicillin (100U/ml), streptomycin (100µg/ml) and L-glutamine (2mM). The cells were then incubated at 37°C and 1% $CO_2$. Bromodeoxyuridine (BrdU) was added to the cell culture medium to prevent proliferation of residual fibroblasts and other non-myocardial cells (Chlopcikova et al, 2001; Simpson & Savion, 1982).

| | | | |
|---|---|---|---|
| Dishes | 10 cm | 12 well/plate | 24 well/plate |
| Cell density | 6-10 millions | 0.5 millions/well | 0.3 millions/well |

| | | |
|---|---|---|
| CBFHH buffer | NaCl | 137 mM |
| | KCl | 5.36 mM |
| | $MgSO_4 \cdot 7H_2O$ | 0.81 mM |
| | Dextrose (± D-Glucose) | 5.55 mM |
| | $KH_2PO_4$ | 0.44 mM |
| | $Na_2HPO_4$ | 0.34 mM |
| | HEPES | 20.06 mM |
| | pH 7.2 | |
| | sterile filtration | |
| Trypsin solution | trypsin | 1.36 mg/ml |
| | DNase solution | 0.8 % (v/v) |
| | Penicillin | 100 U/ml |
| | Streptomycin | 100 µg/ml |
| | CBFHH | |
| | sterile filtration | |
| DNase solution | DNase | 2 mg/ml |
| | NaCl (0.15M) | |

| | | |
|---|---|---|
| 5*Ads buffer | NaCl | 580 mM |
| | HEPES | 100 mM |
| | $NaH_2PO_4$ | 5 mM |
| | Glucose | 28 mM |
| | KCl | 27 mM |
| | $MgSO_4$ | 4 mM |
| | pH 7.35 | |
| | sterile filtration | |
| collagenase solution | collagenase | 158 U/ml |
| | 1*Ads buffer | |
| | sterile filtration | |
| MEM-medium | MEM | 10.5 g |
| | $NaHCO_3$ | 4.2 mM |
| | pH 7.3 | |
| | Vitamin B12 (2mg /ml) | 0.1 % (v/v) |
| | sterile filtration | |
| Preplating medium | FCS | 5% (v/v) |
| | Penicillin | 100 U/ml |
| | Streptomycin | 100 µg/ml |
| | L-glutamine | 2 mM |
| | MEM-medium | |
| Growth medium (NRCM) | FCS | 5% (v/v) |
| | BrdU | 100 µM |
| | Penicillin | 100 U/ml |
| | Streptomycin | 100 µg/ml |
| | L-glutamine | 2 mM |
| | MEM-medium | |

## 3. Cell culture of neonatal rat cardiomyocytes

24 hours after isolation, cell culture medium supplemented with 5% (v/v) FCS, 100µM BrdU, penicillin (100U/ml), streptomycin (100µg/ml) and L-glutamine (2mM) was replaced by culture medium supplemented with 1% FCS instead of 5% (Chlopcikova et al, 2001). Cardiomyocytes were therefore cultured in MEM supplemented with 1% (v/v) FCS, BrdU (100µM), penicillin (100U/ml), streptomycin (100µg/ml), and L-glutamine (2mM) and incubated at 37°C and 1% $CO_2$. 24 hours prior to experiments, NRCMs were incubated in serum-depleted medium.

| | | |
|---|---|---|
| Culture medium (NRCM) | FCS | 1% (v/v) |
| | BrdU | 100 µM |
| | Penicillin | 100 U/ml |
| | Streptomycin | 100 µg/ml |
| | L-glutamine | 2 mM |
| | MEM-medium | |
| Serum-depleted medium (NRCM) | BrdU | 100 µM |
| | Penicillin | 100 U/ml |
| | Streptomycin | 100 µg/ml |
| | L-glutamine | 2 mM |
| | MEM-medium | |

## 4. Isolation of adult mouse cardiomyocytes

Julia Becker isolated adult mouse cardiomyocytes by enzymatic dissociation of the ventricles of eight week-old wild type or Erk2$^{T188S}$ transgenic male mice (cf. Material IV). In comparison to neonatal rat cardiomyocyte isolation, only one animal was used per preparation. The animal was sacrificed by cervical dislocation and the collected heart was digested by Langendorff perfusion using a mixture of collagenase A and D (Roche). Prior to digestion, the blood was washed out with perfusion buffer (3ml/min).

At the end of the digestion the heart was placed on a 60mm culture dish and teased apart. The solution was then filtered through a cell mesh (200µm) to separate cell and tissue debris from the freshly isolated cardiomyocytes. Cells were collected in 5ml stop buffer and let 10 minutes to settle by gravity. The supernatant was then discarded and 10ml of fresh stop buffer were added.

During cell isolation the cell membrane is damaged and become partially permeable. For this reason adult CMs have to be digested in $Ca^{2+}$-free buffer and they should stay in $Ca^{2+}$-free buffer until the damaged membrane is repaired (Woodcock & Matkovich, 2005). For this reason 10µl of $CaCl_2$ (100mM) were added slowly in stop buffer. After 4 minutes 20µl, later 30µl and finally 40µl of $CaCl_2$ (100mM) were incorporated in order to have a final concentration of calcium at 1mM. Cells were either plated on laminin (amsbio)-coated coverslips or separated in 1.5ml reaction tubes in minimum essential medium supplemented with 2mM L-glutamine, 0.1% BSA (Sigma), 1% penicillin/streptomycin, 1xITS Supplement (Sigma) and 20mM 2,3-butanedione monoxime (BDM; Sigma) to prevent contractile activity. 2 hours after plating, cells were either transuded with adenoviruses (cf. Methods IV.2) or stimulated (cf. Methods V.1.a).

| | | |
|---|---|---|
| Perfusion buffer | NaCl | 120 mM |
| | KCl | 15 mM |
| | $Na_2HPO_4$ | 0.6 mM |
| | $KH_2PO_4$ | 0.6 mM |
| | $MgSO_4$-•$7H_2O$ | 1.2 mM |
| | NaHEPES | 10 mM |
| | $NaHCO_3$ | 4.6 mM |
| | Taurine | 30 mM |
| | BDM | 10 mM |
| | Glucose | 5.5 mM |
| | pH 7.4 | |
| | sterile filtration | |
| Stop buffer | FCS | 10% v/v |
| | $CaCl_2$ | 12.5 µM |
| | perfusion buffer | |

| | | |
|---|---|---|
| Digestion buffer | trypsin | 2.5% v/v |
| | $CaCl_2$ | 12.5 µM |
| | collagenase A | 4.4 U |
| | collagenase D | 12.3 U |
| | perfusion buffer | |

# II. BACTERIAL TRANSFORMATION

## 1. Heat shock transformation method

*Competent Escherichia coli* (E. coli) cells, i.e. XL1 blue (Yang, 1992), were transformed with plasmid DNA using the heat shock transformation method (Bergmans et al, 1981). 100µl of competent XL-1 blue cells were mixed with 0.1-1µg DNA and placed on ice for 15 minutes. The mixture was then incubated at 42°C for 50 seconds (heat shock) and cooled down on ice for 1 minute. 900µl of lysogeny broth (LB medium) (Bertani, 1951) without selection antibiotics were added to the bacteria. Bacteria were then incubated at 37°C for 50 minutes. 50 to 100µl of bacteria suspension were plated on LB-agar plates supplemented with ampicillin and incubated overnight at 37°C.

| | | |
|---|---|---|
| LB-medium | peptone from casein | 10 g/l |
| | yeast | 5 g/l |
| | NaCl | 10 g/l |
| | distilled water | |
| | autoclave | |
| LB-plates | agar | 10 g/l |
| | ampicillin | 0.01 % w/v |
| | LB-medium | |

## 2. Electroporation

*Competent Escherichia coli* (E. coli) cells, i.e. DH10B (Grant et al, 1990) were transformed with plasmid DNA using the electroporation. A large electric pulse (2500V) disturbs temporarily the cell membrane, allowing DNA to enter the cell (Neumann et al, 1982). This method was used after BP and LR reactions (cf. Methods III. 3).

50µl DH10B cells were transformed with the BP or LR reaction mixture by electroporation with a MicroPulse™ BioRad (2500V). The bacteria were then incubated for 30 minutes in 200µl of LB-medium without antibiotics. 150µl of the cell solution were then platted on LB-plates supplemented with kanamycin (after

the BP reaction) or with ampicillin (after the LR reaction) and incubated overnight at 37°C.

### 3. **Culture of E. Coli**

Single colonies were picked and grown overnight at 37°C in LB-medium supplemented with ampicillin or kanamycin (0.01% w/v).

### 4. **Extraction of plasmid DNA**

Plasmid DNAs were isolated from the transformed bacteria following the instructions of Qiagen “Plasmid DNA Purification Using Qiagen Plasmid Plus Midi or Maxi Kits”.
Extracted plasmid DNAs were then purified (cf. Methods III.2), controlled by PCR or sent for sequencing (eurofins MWG)

# III. PREPARATION OF RECOMBINANT ADENOVIRUS VECTOR

## 1. Polymerase chain reaction (PCR)

For transient transfection of neonatal rat cardiomyocytes recombinant adenoviruses were generated (Bueno et al, 2000; Merkle et al, 2007). The respective DNA construct was cloned into pDONOR via PCR and then enzymatically inserted into the adenoviral vector pAD-CMV-V5-Dest (Invitrogen).
The polymerase chain reaction (PCR) is a specific method used to amplify a specific region of deoxyribonucleic acid or to insert specific mutations. For this amplification strand-specific oligonucleotides (primers) are used. Their sequences consist of 18-21 base pairs and are complementary to the flanking area of the respective, desired DNA sequence. The amplification follows a three-step reaction cycle: denaturation of the template DNA, hybridization of oligonucleotides (annealing) and DNA polymerization (elongation). This reaction takes place in a thermocycler.

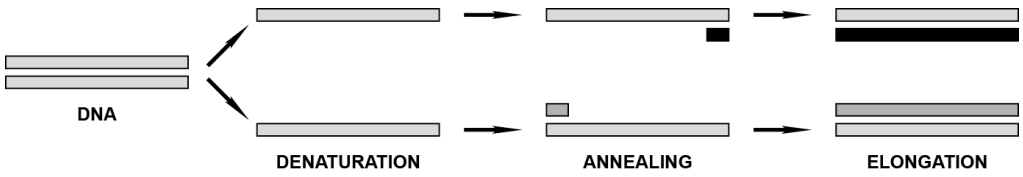



**Figure 8. Principle of the PCR.** The polymerase chain reaction (PCR) is a specific method used to amplify a specific region of deoxyribonucleic acid or to insert specific mutations. During the denaturation, the double stranded DNA dissociates in two single stranded DNAs. Forward and reverse primes bind to the single strands, annealing, and bases (dNTPs) are added complementary to the strands (elongation). Denaturation, annealing and elongation are the three steps of the PCR-cycle.

| | | |
|---|---|---|
| PCR reaction mixture: | 0.1-1 µg | template |
| | 0.5-2 µM | primer forward (cf. Material VII.3) |
| | 0.5-2 µM | primer backward (cf. Material VII.3) |
| | 200 µM | d-NTPs |
| | in 1×DNA-Polymerase buffer | |
| | 1 U | DNA-Polymerase |

The PCR (Figure 8) was performed in a BioRad S1000 Thermal Cycler, with the following conditions:

| Temperature (°C) | Time | Number of cycle | Step |
|---|---|---|---|
| 94 | 3-5 | 1× | for denaturation |
| 94<br>60<br>72 | 1<br>1<br>2 | 30× | denaturation<br>annealing<br>elongation |
| 72 | 7 | 1× | end of elongation |

PCRs are not only used to insert specific mutations, they are also used to control whether transformed bacteria express a specific gene of interest. The same protocol as the one previously described is followed except that templates for this "control PCR" correspond to 5µl of bacterial culture. Control PCRs are a useful method to screen a large amount of bacteria in order to select the ones that express the gene of interest. DNA is then extracted only from those specific bacteria (cf. Methods II.4).

## 2. **Agarose-gel electrophoresis and DNA purification**

PCR products or DNA fragments were separated on on 1%-2% (w/v in TAE running buffer) agarose gels supplemented with 0.1µl/ml ethidium bromide. DNA samples were loaded on the gels in TAE running buffer and a DNA ladder (BioLabs) was used to define the size of the DNA fragments. The electrophoresis ran at 100V with TAE running buffer. Separated DNA fragments were detected under under UV-light by fluorescence of ethidium bromide. The band of interest was excised from the gel and the DNA was extracted and purified following the protocol of QIAquick Gel Extraction Kit.

| | | |
|---|---|---|
| 50*TAE buffer | EDTA | 0.1 M |
| | acetic acid | 1 M |
| | Tris, pH 8 | 2 M |
| | distillated water | |

| | | |
|---|---|---|
| TAE running buffer | 50*TAE buffer<br>distillated water | 2% v/v |
| ladder buffer | EDTA (0.5M, pH 8)<br>glycerin (85%)<br>bromophenol blue<br>distilled water | 100 mM<br>50% (v/v)<br>0,1% (v/v) |

## 3. **Adenoviruses preparation**

For transient transfection of neonatal rat cardiomyocytes Nadine Yurdagül-Hemmrich generated adenoviruses using the Gateway system (Invitrogen).
The gateway system is based on the site-specific recombination system used by phage λ to introduce its DNA in bacteria. In both organisms there is a stretch of DNA called attB (for bacteria) or attP (for phage). When the phage infects the bacterium, the phage DNA recombines with the bacterial DNA via the att sites. After recombination of attB and attP sites the hybrid sides are called attL and attR. This phenomenon is the base of the enzymatic BP and LR reactions used in the Gateway system.
The DNA of interest was amplified via PCR with primers that included artificial attB restriction sites (cf. Material VII.3). After purification the PCR product was enzymatically inserted into the donor vector pDON221, which is flanked with to attP sites, following Invitrogen protocols. Upon addition of BP-clonase the att sites recombined and formed the entry clone (BP reaction). For this insertion reaction, the reaction mix was incubated for 1 hour 45 minutes at 25°C. The reaction was stopped by addition proteinase K (Invitrogen) and subsequent incubation for 10 minutes at 37°C.
50µl DH10B (E. coli) were then transformed with the BP reaction mixture by electroporation (cf. Methods II.2). Plasmid DNAs were then isolated, controlled by PCR and sent for sequencing (eurofins MWG).

The DNA-construct was then transferred from pDON221 vector (entry clone) to pAD-CMV-V5-Dest. vector (destination clone) by another enzymatic reaction the LR reaction (Invitrogen). The entry clone freshly obtained was flanked with two attL sites while the pAD-CMV-V5-Dest. Vector had two attR sites. Upon addition of LR-clonase all and attR sites recombined and created the destination clone.

For this enzymatic reaction, the reaction mixture was incubated for 3 hours at 25°C. The reaction was stopped by incubation with proteinase K (Invitrogen) for 10 minutes at 37°C.
50µl DH10B (E. coli) were transformed with the LR reaction mixture by electroporation (cf. Methods II.2). Plasmid DNA was isolated and controlled by PCR.

The pAD-CMV-V5-Dest. vector with the DNA of interest was then digested with PacI for 4 hours at 37°C. DNA was then precipitated with sodium acetate (3M) and stored at -80°C in ethanol (100%).
The following day, the mixture was centrifuged for 15 minutes (14000rpm; 4°C) and the supernatant was discarded. 300µl of ethanol (70%) were added to the pellet and the solution was centrifuged for 3 minutes (14000rpm; 4°C). Finally, ethanol was removed and DNA was dissolved in 20µl $H_2O$.
AD-HEK cells, previously split in 10cm culture dishes, were then transfected with the linearized DNA construct by effectene (Qiagen) (cf. Methods IV.1.c). The cells were incubated at 37°C and 5% $CO_2$ for up to 7-14 days till the cells detached from cell culture dishes. The cells and media were collected and centrifuged for 10 minutes (850rcf; RT). The supernatant was discarded and the cell pellet was resuspended in 1 ml of sterile Tris buffer (10mM, pH 8.0).
The cell suspension was frozen in liquid nitrogen and subsequently thawed in a water-bath (37°C) three times. 500µl of the virus amplification was added to freshly split AD-HEK cells. Following the same protocol viruses were then amplified two further times. After the 3$^{rd}$ amplification, viruses were operational and aliquots were stored at -80°C.

# IV. TRANSFECTION METHODS

## 1. Transfection methods in established cell line

### a. Calcium phosphate transfection method

Calcium phosphate transfection method was used for transient transfection in human embryonic kidney (HEK293) cells (Chen & Okayama, 1987; Jordan et al, 1996). Cells were split into 10cm cell culture dishes and were transfected after 3 to 6 hours. Following 1ml transfection mix was used per 10cm cell culture dish: 3-10µg were diluted with sterile water to 450µl (usually 5µg of the Erk2 constructs and 3µg of the corresponding receptors were used). 50µl of $CaCl_2$ (2.5M) and 500µl of 2*BBS buffer were added to the DNA. The mixture was vigorously shaken and incubated for 15 minutes at room temperature to achieve a complete precipitation of the DNA-CaPO4 complex. The precipitate was then added drop-wise onto the cells. HEK293 cells were then incubated 48h at 37°C and 5% $CO_2$.

| | | |
|---|---|---|
| 2*BBS buffer | BES | 50 mM |
| | NaCl | 280 mM |
| | $Na_2HPO_4 \bullet 2H_2O$ | 1.5 mM |
| | adjust the pH 6.95 with NaOH (5M) at 20°C | |
| | sterilization | |

### b. DEAE transfection method

Diethylaminoethyl-Dextran (DEAE-Dextran) method was used in monkey kidney cells (COS-7) for transient transfection (Calos et al, 1983). COS7 cells were split in 6 well/plates on day prior to transfection. Cells were then washed once with TBS-buffer and exposed to DNA/DEAE-dextran mixture for 15 minutes. 400µl of the following mixture were used per well. In a sterile 1.5ml reaction tube 2.5µg DNA (receptors) and 6.5µg DNA (Erk-mutants) were diluted in 500µl of TBS-buffer and mixed with 500 µl of DEAE-Dextran (1mg/ml of TBS-buffer).

15 minutes latter, fresh DMEM medium supplemented with chloroquine (10µM) and 10% (v/v) FCS was added on the cells. After a three-hour incubation at 37°C and 5% $CO_2$, the medium was sucked off and COS-7 cells were treated with 10% (v/v) DMSO for exactly one minute. Cells were then incubated at 37°C by 7% $CO_2$ with fresh DMEM medium supplemented with 10% (v/v) fetal calf serum

(FCS), penicillin (100U/l), streptomycin (100µg/ml) and L-glutamine (2mM). Experiments were performed 24-48 hours after transfection.

| | | |
|---|---|---|
| TBS-buffer | Tris | 25 mM |
| | NaCl | 137 mM |
| | KCl | 5 mM |
| | MgCl2 | 0.5 mM |
| | CaCl2 | 0.7 mM |
| | Na2HPO4 | 0.6 mM |
| | pH 7.4 | |

**c. Effecten transfection method**

For virus amplification (cf. Methods III.3) AD-HEK cells were transfected with the effecten method (Qiagen). One day prior to transfection AD-HEK cells were split in 10cm culture dishes. Following the protocol given by Qiagen, the linearized DNA construct was mixed with EC-buffer and the enhancer. After 5-minute incubation at RT effecten was added and the solution rested 10 more minutes. The effecten solution and freshly DMEM medium were added on the cells.

## 2. Adenoviral infection

Adenoviruses were used for infection of neonatal rat cardiomyocytes (NRCMs) and adult mouse cardiomyocytes (CMs) (Bueno et al, 2000; Merkle et al, 2007). 2 hours (CMs) or 48 hours (NRCMs) after cell isolation, cells were infected with adenoviruses encoding for proteins that needed to be analyzed. The transfection method was optimized for equal expression of the constructs that needed to be compared. The culture medium was exchanged 24 hours after transfection to serum-depleted medium for another 24 hours. Transfection efficiency was controlled by Western blot analysis.

| | | |
|---|---|---|
| Culture medium(NRCM) | 1% (v/v) FCS | 1% (v/v) |
| | BrdU | 100 µM |
| | Penicillin | 100 U/ml |
| | Streptomycin | 100 µg/ml |
| | L-glutamine | 2 mM |
| | MEM-medium | |
| Serum-depleted medium (NRCM) | BrdU | 100 µM |
| | Penicillin | 100 U/ml |
| | Streptomycin | 100 µg/ml |
| | L-glutamine | 2 mM |
| | MEM-medium | |

# V. LYSATES AND WESTERN BLOTS

## 1. Protein lysate preparation

### a. Protein lysates from HEK293, NRCMs, adults CMs and mouse hearts

NRCMs were cultivated in 12 well/plates at a density of 0.5 million cells/well. After 24 or 48 hours of cultivation, cells were starved in serum-depleted media.followed by the treatment with isoproterenol (5µM), phenylephrine (5µM), angiotensinII (100nM), carbachol (10µM) or forskolin (30µM) for either 10 minutes or 24 hours. When indicated, cells were pretreated with PD98059 (30µM; 90 minutes; Erk inhibitor (Teos et al, 2008)), *pertussis toxin* (0.1µg/ml; 20 hours; $G\alpha_i$ inhibitor (Morisco et al, 2001; Xiao et al, 1999; Zheng et al, 2010)) or KH7 (30µM; 30 minutes; adenylyl cyclase inhibitor (Kumar et al, 2009; Salinthone et al, 2011)) prior to isoproterenol treatment. For long-term stimulation, culture medium was supplemented with ascorbic acid (100µM) to avoid degradation of isoproterenol. After stimulation, cells were lysed with 150µl of ice-cold lysis buffer supplemented with phenylmethylsulfonyl fluoride (PMSF), benzamidin and soybean, which are phosphatase and protease inhibitors (Kunitz, 1946), for 10 minutes at 4°C. Lysates were then collected in fresh 1.5ml reaction tubes, 50µl Laemmli buffer (Roti®-load1, 4*concentrated, Roth) was added and samples were sonicated and stored at -20°C.The same protocol was used to prepare lysates from HEK293 cells or to determine the transfection efficiency of NRMCs.

Direct after cell isolation, adult cardiomyocytes were separated equally in fresh 1.5ml reactions tubes. They were then incubated with isoproterenol (5µM) at 37°C. After 6 minutes CMs were shortly centrifuged and the supernatants were discarded. 100µl of PBS and 100µl Laemmli buffer (Roti®-load1, 4*concentrated, Roth) were added and samples were stored at -20°C.

Similarly, left ventricles from transgenic (T188T or T188S) and wild-type mice were homogenized in 700µl lysis buffer with an Ultra-turrax. Homogenized heart lysates were then centrifuged for 3 minutes at 1000 rcf at 4°C and supernatants were collected in fresh 1.5ml reaction tubes. Potein concentration was

determined by BCA-protein assay and then Laemmli buffer (Roti®-load1, 4*concentrated, Roth) was added.

| | | |
|---|---|---|
| Lysis buffer | 2*TSE (4°C) | 50% (v/v) |
| | 10*IBx | 10 % (v/v) |
| | Triton X-100 | 1% (v/v) |
| | distillated water | |
| 2*TSE | Tris-HCl | 100 mM |
| | NaCl | 600 mM |
| | EDTA | 10 mM |
| | $NaN_3$ | 0.02% (w/v) |
| | adjust the pH 7.4 | |
| 10*IBx | NaF | 500 mM |
| | $Na_4P_2O_7$ | 50 mM |
| | $Na_3VO_4$ | 1 mM |
| | $NaN_3$ | 0.02% (w/v) |
| Protein inhibitor (PI) | soybean/trypsin inhibitor | 2 mg/ml |
| | benzamidin | 6 mg/ml |
| | Tris (50 mM) pH 7,4 | |
| Phenylmethylsulfonyl fluoride | PMSF | 17.4 mg/ml |
| | Ethanol | |

**b. Quantification of protein concentration**

Protein concentrations were determined by colorimetric detection using Pierce® BCA Protein Assay Kit (Thermo Scientific). Eight standards with known concentration (from 25 to 2000µg/ml) of Albumine (BSA) and a blank were prepared. Each standard and unknown sample was analyzed in triplicates (3*25µl) on 96 well/plates in BCA working reagent (200 l). After 20 minute incubation at 37°C, absorbance was measured at 562nm by a spectrophotometer SpectraMax340 (Molecular Device services).

The wavelength measurement of each BSA standard was plotted on a standard curve versus its concentration. The protein concentration of each unknown sample was determined using this standard curve.

## 2. Western blot analysis

### a. SDS- Polyacrylamide gel electrophoresis (SDS-PAGE)

The gel electrophoresis is a technic used to separate and characterize molecules (Jensen, 1965; Sogami & Foster, 1962). Two polymers, agarose or polyacrylamide are used to prepare the gel. Large molecules such as DNA are separated on agarose gels (cf. Methods III.2) while smaller ones such proteins are isolated on polyacrylamide gels. TEMED and APS initiate the polymerization of the gel. SDS gels are composed of two gels: the upper/stacking gel, where the molecules get focused and the lower/running gels, where they are separated.

Laemmli buffer (Laemmli, 1970) is added to the sample in order to denaturize the proteins. Denaturized proteins are then separated from each other regarding their size and not their shape. SDS or sodium dodecyl sulfate, presents in laemmli buffer, is a detergent that denaturized the proteins and coat them with negative charges. Under current application negative charged proteins migrate to the positive charged electrode through the gel. The proteins are then separated depending on their molecular weight, since the smallest proteins migrate faster. The laemmli buffer stains the samples blue thanks to bromophenol blue, which serves as an indicator dye and facilitates the detection of the proteins while they migrate.

After protein denaturation, which was enhanced by warming the protein 20 minutes at 40°C, samples were loaded into the wells of the gel and were covered with SDS buffer. The protein marker III (Peqlab) was used as standard and 70V were applied per gel.

To prepare 12% SDS-gel:

| | | |
|---|---|---|
| Lower/running gel | lower buffer | 25% v/v |
| | Aa/bis | 40% v/v |
| | ("30"= 30 % Acrylamid, 0,8% Bisacryl) | |
| | TEMED | 0.05% v/v |
| | APS (10 % (w/v)) | 0.655 v/v |
| | (Ammoniumperoxidisulfat) | |
| | distillated water | |
| Upper/stacking gel | upper buffer | 25% v/v |
| | Aa/bis | 12.5% v/v |
| | ("30"= 30 % Acrylamid, 0,8% Bisacryl) | |
| | TEMED | 0.1% v/v |
| | APS (10 % (w/v)) | 1% v/v |
| | (Ammoniumperoxidisulfat) | |
| | distillated water | |
| Lower buffer | SDS | 14 mM |
| | Tris (3M), pH 8.8 | 50% v/v |
| | distilled water | |
| Upper buffer | SDS | 14 mM |
| | Tris (1M) pH 6.8 | 50 % v/v |
| | distilled water | |
| 10*SDS buffer | Tris | 250 mM |
| | Glycin | 2 M |
| | SDS | 35 mM |
| | distilled water | |

### b. Protein transfer from SDS gels to PVDF membranes

After separation of the proteins by SDS gel electrophoresis, proteins were transferred on protein binding membrane (PVDF membrane) in Transfer-Blot® SD Semi-Dry electrophoretic transfer cell (BioRad) for one hour and 30 minutes at 15V. Prior to transfer, membranes were activated in methanol and blotting papers were bathed in transfer buffer. For the transfer, the gel was embedded in a sandwich of blotting paper, gel, PVDF membrane and another layer of blotting paper (Burnette, 1981; Matsudaira, 1987).

| | | |
|---|---|---|
| transfer buffer | Tris | 2 mM |
| | Glycin | 150 mM |
| | Methanol | 20% v/v |
| | distillated water | |

### c. Western blot detection

The western blot detection is a well-known method (Burnette, 1981). After protein transfer onto PVDF membranes, unspecific binding sites on the membranes were blocked by 2 hours of incubation in blocking milk buffer at room temperature. Primary antibodies were added for overnight incubation at 4°C diluted as shown below (cf. table 1). Membranes were then washed 5*15 minutes either with BSA or with 1*PBS (+ Tween) as mentioned below (cf. table 1). Membranes were then exposed to secondary antibodies at room temperature (cf. table 1). After 2 hours, membranes were washed again with buffers as indicated in the table below.

To reveal our target-proteins, membranes were exposed few minutes to a scintillating solution either luminol or ECL Plus Western Blotting Detection system (GE Healthcare). Signals were visualized and documented with Fuji medical X-ray films

| | | |
|---|---|---|
| Blocking milk | NaCl | 100 mM |
| | milk powder | 5 % (w/v) |
| | Tris (1M, pH 7) | 10 mM |
| | Tween 20 | 0.1% (v/v) |
| | distillated water | |

| | | |
|---|---|---|
| BSA | NaCl (2M) | 150 mM |
| | Tris (1M, pH 7,3) | 50 mM |
| | NP40 | 0.2% (v/v) |
| | albumine (fraction V) | 0.25% (w/v) |
| | distillated water | |
| PBS (+ Tween) | Tween 20 | 0.1 % (v/v) |
| | 1*PBS | |
| 10*PBS | NaCl | 1.37 M |
| | KCl | 27 mM |
| | $Na_2HPO_4 \bullet 7H_2O$ | 11 mM |
| | $KH_2PO_4$ | 15 mM |
| | distillated water | |

For luminol development solutions A and B were mixed in the dark. P-Cumarsäure (90mM) and luminol (250mM) were dissolved in DMSO and stored at -20°C.

| | | |
|---|---|---|
| solution A | p-Cumarsäure (90 mM) | 55 µl |
| | luminol (250 mM) | 125 µl |
| | Tris (100 mM, pH 8,3) | 12.5 ml |
| solution B | $H_2O_2$ (30 % w/v) | 7.5 µl |
| | Tris (100 mM, pH 8.3) | 12.5 ml |

| primary antibodies | secondary antibodies | wash buffer | Development |
|---|---|---|---|
| **anti-HA**<br>1:4000 in milk | goat-anti-mouse<br>1:4000 in BSA | BSA | ECLplus (GE Healthcare) |
| **anti-Flag**<br>1:4000 in milk | goat-anti-mouse<br>1:4000 in BSA | 1*PBS + 0.1 % (v/v) Tween<br>1*PBS | luminol |
| **anti-Gβ**<br>1:4000 in milk | goat-anti-rabbit<br>1:4000 in BSA | BSA | ECLplus (GE Healthcare) |
| **anti-Erk1/2 p44/42**<br>1:1000 in BSA with 750µM $NaN_3$ | goat-anti-rabbit<br>1:4000 in BSA | 1*PBS + 0.1% (v/v) tween<br>1*PBS | luminol |
| **anti-phospho Erk1/2**<br>1:1000 in BSA with 750µM $NaN_3$ | goat-anti-rabbit<br>1:4000 in BSA | 1*PBS + 0.1% (v/v) tween<br>1*PBS | luminol |
| **anti-phospho-Erk1/2$^{Thr188}$**<br>1:2000 in BSA with non phosphorylated peptides (Lorenz et al, 2009a) | goat-anti-rabbit<br>1:4000 in BSA | BSA | ECLplus (GE Healthcare) |

**Table 1.**

# VI. CO-IMMUNOPRECIPITATION ASSAYS

Interaction of Gβγ-subunits and Erk2 was determined by co-immunoprecipitation assays either in HEK293 cells or in NRCMs (Lorenz et al, 2009a). Protein A-Sepharose beads (8µL/sample) were washed three times with PBS and then incubated overnight with either HA- or Flag-tag antibodies (0.6µL/sample, HEK293; 1µl/sample, NRCM), or Erk1⁄2 p44/42 antibodies (6µL/sample) and washed again with PBS to remove residual unbound antibodies.

NRCMs were cultivated in 10cm cell culture dishes at a density of 6-10 million cells/plate. After 24-hours of starvation in serum-depleted MEM-media, cells were incubated without or with isoproterenol (NRCMs, 5µM, 24 hours; HEK293, 1µM, 10 minutes) or phenylephrine (2µM). NRCMs were lysed in ice-cold NP40-lysis buffer supplemented with phenylmethylsulfonyl fluoride (PMSF), benzamidin and soybean, which are phosphatase and protease inhibitors (Kunitz, 1946). Cells were then centrifuged for 10 minutes (14000rpm; 4°C). Supernatants were transferred to antibody labeled-Protein A Sepharose beads. After 2 hours of incubation by rotation at 4°C, beads were washed three times with ice-cold PBS. 25µl of Laemmli buffer (Roti®-load1, 4*concentrated, Roth) were added and samples were stored at -20°C.

| | | |
|---|---|---|
| NP40-lysis buffer | NP40 | 0.5 % (v/v) |
| | NaCl | 150 mM |
| | $Na_4P_2O_7$ | 25 mM |
| | $Na_3VO_4$ | 100 µM |
| | Natriumglycerophosphate | 50 mM |
| | NaF | 50 mM |
| | EDTA | 2 mM |
| | EGTA | 2 mM |
| | Tris pH 8.0 | 25 mM |
| | Glycerin | 10 % (v/v) |
| Protein inhibitor (PI) (100x) | soybean/trypsin inhibitor | 2 mg/ml |
| | benzamidin | 6 mg/ml |
| | Tris (50 mM) pH 7,4 | |

| | | |
|---|---|---|
| Phenylmethylsulfonyl fluoride (100x) | PMSF<br>Ethanol | 17.4 mg/ml |

# VII. [$^3$H]-ISOLEUCINE INCORPORATION ASSAYS

To determinate the hypertrophic response of NRCMs to isoproterenol or phenylephrine treatment, protein synthesis rates in cells were measured by [$^3$H]-isoleucine incorporation (Lorenz et al, 2009a). NRCMs were cultivated in poly-lysine pre-coated 24-well plates at density of 0.3 million cells/well. After 48 hour of culture, cells were infected with adenoviruses (cf. Methods IV.2) and 24 hours later, cells were starved in serum-depleted medium for 24 hours. Cells were then incubated with serum-free medium supplemented with [$^3$H]-isoleucine (1µCi), ascorbic acid (100µM) and with or without isoproterenol (5µM), phenylephrine (2µM) or forskolin (30µM). If indicated, cells were pretreated with PD98059 (30µM, 90 minutes; Erk inhibitor (Teos et al, 2008)), *pertussis toxin* (0.1µg/ml; 20 hours; $G\alpha_i$ inhibitor (Morisco et al, 2001; Xiao et al, 1999; Zheng et al, 2010)) or KH7 (30µM; 30 minutes; adenylyl cyclase inhibitor (Kumar et al, 2009; Salinthone et al, 2011) prior to isoproterenol treatment.

After 24 hours of incubation, labeled NRCMs were washed twice with 1*PBS and protein was precipitated with 500µl ice-cold TCA [5% (w/v)] for 1 hour at 4°C. Precipitated proteins were then dissolved in 500µl NaOH (0,5M) for at least 30 minutes at 37°C and Lumasafe$^{TM}$plus was added. Incorporated [$^3$H]-isoleucine was quantified in a scintillation β-counter.

# VIII. MICROSCOPE ANALYSES

## 1. Cell fixation

For confocal microscopic analyses, NRCMs, adult mouse CMs and COS-7 cells were washed three times with 1*PBS buffer and fixed with 4% (w/v) paraformaldehyde solution (10min, RT). After fixation, cells were rinsed twice with PBS and stored at 4°C for further analyses.

PFA 4% preparation: 8g of paraformaldehyde (PFA) were heated to 60°C in 100ml water. NaOH (1M) was dropped into the solution until PFA was dissolved. After the solution has cooled down to room temperature, 2*PBS was added up to 200ml and pH was adjusted to pH 7.4. The solution was aliquoted and stored at -20°C.

## 2. YFP-tagged constructs

For visualization of the distribution of the protein within the cells by confocal microscopy, the Erk2 constructs were N-terminally tagged with the yellow fluorescent protein (YFP). Monkey kidney cells (COS-7) were transfected with either YFP-Erk2 (T188T) or YFP-Erk2$^{T188A}$ (T188A) and $\beta_2$-adrenergic receptors using the DEAE-Dextran method (cf. Methods IV.1.b). Neonatal rat cardiomyocytes and adults mouse CMs were infected with adenoviruses encoding for GFP, YFP-Erk2 (T188T) or YFP-Erk2$^{T188A}$ (T188A) (cf. Methods IV.2). After stimulation of the cells with isoproterenol (5µM; 10 minutes, COS7 cells; 12 hours, adults CMs; 24 hours NRCMs), cells were fixed in paraformaldehyde solution [4% (w/v)] (cf. Methods VIII.1). Nuclei were then stained with DAPI (cf. Methods VIII.3.c).

## 3. Staining methods

### a. Phalloidin staining

During cardiomyocyte hypertrophy, actin synthesis increases and actin filaments reorganize (Aoki et al, 1998; Dorn et al, 2003). Actin filaments were stained with Alexa Fluor® 488 labeled phalloidin and analyzed cytoskeletal reorganization by

confocal microscopy. This method was also used to quantify cell surface area of neonatal cardiomyocytes before and after isoproterenol treatment.

Neonatal rat cardiomyocytes were cultivated in 12-well plates for 2 days and were then infected with adenoviruses encoding for either GFP, Flag-Erk2 (T188T), Flag-Erk2$^{T188S}$ (T188S) or Flag-Erk2$^{T188A}$ (T188A). Cells transfected with GFP, referred as mock transfected cells, served as controls. 24 hours later, cells were starved in serum-depleted medium. After 24 hours of starving, NRCMs were treated without or with isoproterenol (5µM) in medium supplemented with ascorbic acid (100µM) for another 24 hours and then fixed with 4% (w/v) paraformaldehyde (cf. Methods VIII.1). To permeabilize the cell membrane, cells were covered with a -20°C cold acetone/methanol solution (1:1) for 2 minutes. Unspecific binding sites for phalloidin were then blocked using a solution of 2% (w/v) BSA dissolved in PBS. After 30 minutes, NRCMs were incubated with Alexa Fluor® 488 labeled phalloidin (5 U/ml) for 20 minutes, at room temperature in the dark.

**b. α-actinin staining**

To determine the purity of the NRCM preparation, NRCMs were stained with antibodies directed against α-actinin. These antibodies were used to detect the cardiomyocyte specific a-actinin striation.

Neonatal rat cardiomyocytes were cultivated in 6 well/plates for two days and fixed with 4% (w/v) paraformaldehyde (cf. Methods VIII.1). After permeabilization with -20°C cold acetone/methanol solution (1:1) and blockage of unspecific binding sites with BSA-buffer, NRCMs were incubated with antibodies directed against α-actinin (1:400) for one hour at room temperature. Cells were then washed three times with PBS and Cy3-coupled anti-mouse antibodies (1:800) were added for 20 minutes in the dark. Antibodies were diluted in BSA-buffer (cf. Methods VIII.2.a). Cell nuclei were counterstained with DAPI (cf. Methods VIII.2.c).

**c. DAPI staining**

When indicated, cell nuclei were stained with DAPI. Cells were incubated in the dark with DAPI (700ng/ml). After 30 minutes, cells were washed two times with PBS and were then stored at 4°C in PBS.

## 4. **Confocal microscopy**

Cellular distribution of YFP-Erk constructs, actin rearrangements and changes in cell surface area were detected using a Leica TCS SP5 confocal microscope with a 63*1.4 oil objective. YFP, Alexa Fluor 488 and Cy3 were exited with an argon laser at 514nm (YFP) or at 496nm (Alexa Fluor 488 and Cy3). DAPI was exited with a diode laser at 405 nm. Fluorescence intensities were measured from 550 to 620nm (YFP), from 540 to 600nm (Alexa Fluor 488), from 550 to 600nm (Cy3) and from 430 to 453nm (DAPI). Settings for recording images were kept constant at 400Hz and 1024*1024 pixel format. The standard Leica LAS AF software was used for recording images. Confocal analyses were performed in a blind-fashion and results were then analyzed with ImageJ and Photoshop.

# IX. TRANSGENIC MICE MODEL

## 1. Genotyping of transgenic mice

Genotyping was mostly performed by Martina Fischer. DNA of tails tips was extracted by the addition of 500µl of 50mM NaOH followed 45 minutes of heating at 95°C. To stop the reaction, 60µl of a Tris/EDTA (1M Tris (pH 8) and 10mM EDTA) were added. Supernatant, which contained extracted DNA, was used to perform polymerase chain reaction (PCR) and to identify the mice's genotype. 3µl of supernatant were mixed with 23µl of PCR reaction mixture.

PCR reaction mix/sample:
- 2µl dNTPs (1.25mM)
- 1,25 µl primers (10pmol/µl)
- 0.75 µl $MgCl_2$
- 12.4 µl $H_2O$
- 2.5 µl 10* Taq Polymerase buffer
- 0.35 µl Taq DNA-Polymerase

Four different primers were used for the genotyping strategy (cf. Material VII.1). The first primer attached the $\alpha$-*Mhc* promoter (RF), which controls the Erk constructs (Lorenz et al, 2009a) and the second primer bound Erk (EB). An internal control was performed with the myosin housekeeping gene using two more primers (VF and VB).

The PCR was performed in a BioRad S1000 Thermal Cycler under following conditions:

| Temperature (°C) | Time | Number of cycle | Step |
|---|---|---|---|
| 94 | 60 | 1 | for denaruration |
| 94<br>58<br>72 | 20<br>25<br>30 | 40 | denaturation<br>annealing<br>elongation |
| 15 | 300 | 1 | end of elongation |

PCR-products were then eluted on a 2% (w/v) agarose gel and results were analyzed under UV-light.

| | | |
|---|---|---|
| 50*TAE buffer | EDTA | 0.1 M |
| | acetic acid | 1 M |
| | Tris, pH 8 | 2 M |
| | distillated water | |
| ladder buffer | EDTA (0.5M pH 8) | 100 mM |
| | glycerin (85%) | 50% (v/v) |
| | bromophenol blue | 0.1% (v/v) |
| | distillated water | ad 100 ml |

## 2. **Care of the animals**

Care of the animals (cf. Material IV) was taken in accordance with the Committee on Animal Research of the regional government (Regierung von Unterfranken, Germany).
Julia Becker and Kristina Lorenz organized and performed mouse breeding and tail biopsis. A maximum of 5 mice or 4 rats, respectively shared one cage and a twelve-hour light-dark cycle was provided. The animals had free access to drinking water and appropriate food at any time.

# X. PHYSIOLOGICAL METHODS

## 1. Osmotic mini-pump model

To induce β-adrenergically mediated cardiac hypertrophy *in vivo,* osmotic minipumps (Alzet, 1002) containing isoproterenol (30mg/kg body weight/day) were implanted in 8-week old wild type (FVB/N background) and transgenic mice overexpressing $Erk2^{T188T}$ and $Erk2^{T188S}$ for 14 days (Cha et al, 2010; Leenen et al, 2001; Lorenz et al, 2009a). Minipumps were inserted subcutaneously and during surgery the animals were anesthetized using ketamine (150µg/g) and xylazine (0.5µg/g). Before and after minipump implantation echocardiography was performed by Kristina Lorenz.

## 2. Echocardiography

Cardiac hypertrophy and heart function were analyzed by echocardiography using the Vevo2100 high resolution imaging system (VisualSonics) and a 30-MHz probe. For mouse anesthesia pentobarbital (35mg/kg) was injected intraperitoneally. To preserve body temperature, the animals were placed on a heated platform (42°C).

# XI. HISTOLOGICAL METHODS

Marianne Babl prepared tissue sections from mouse hearts and the histological H&E or Sirius red stainings.

## 1. Tissue preparation for paraffin sections

Ventricles of mouse hearts were fixed in 4% (w/v) paraformaldehyde (PFA) (cf. Methods VIII.1). The tissues were dehydrated in isopropanol solutions with increasing isopropanol concentrations (30%, 50%, 70%, 85%, 95% and 100%) and at the end in xylol. The dewatered ventricles were dived in liquid paraffin and casted in blocks. Transverse heart sections of 2μm were prepared using a Leica RM 2165 microtome and were mounted on microscope slices pre-coated with polylysin. These sections were then used for histological analyses.

## 2. Hematoxylin and eosin (H&E) staining

To determine hypertrophy of individual cardiomyocytes, heart sections were stained with hematoxylin and eosin (Ennis et al, 2003; Lorenz et al, 2009a; Werely, 1976). Slices were first dewaxed in Rotihistol and ethanol solutions with decreasing ethanol concentrations (100%, 90%, 70% and 50%). For each dewaxing step the sections remained for 4 minutes in the corresponding solution. They were then incubated ten minutes with hematoxylin, washed under tap water for 10 minutes and finally placed in eosin solution for two minutes. Hematoxylin stains cell nuclei in blue and eosin stains the cytoplasm in red. Sections were dehydrated with increasing ethanol concentrations (50%, 70%, 90% and 100%), transferred in Rotihistol and embedded with Eukitt (Fluka).
Slices were then photographed with a Leica DM4000B microscope (40×0.75).

## 3. Sirius Red staining

To determine the cardiac fibrosis, heart sections were stained with Sirius Red (Lorenz et al, 2009a; Seeland et al, 2007). Sirius Red staining is used for histological visualization of collagen I and III (Junqueira et al, 1978). Tissue sections were dewaxed as described (cf. Methods XI.2), stained in a Sirius Red solution for 45 minutes and then dehydrated and embedded with Eukitt (Fluka).

| | | |
|---|---|---|
| Sirius Red solution | 1% of Direct Red 80 (Aldrich) solution | 1 fraction |
| | saturated solution of picric acid | 9 fractions |

## 4. Immunohistochemistry

To detect the activation, i.e. phosphorylation, of Elk1, which is a nuclear target of Erk1/2, immunohistochemistry of heart sections was performed using an antibody directed against pElk1 (Cell Signaling, 9182) (Lorenz et al, 2009a; Subramanian et al, 2002). Histological heart tissue slices were dewaxed in Rotihistol and ethanol solutions with decreasing ethanol concentrations (100%, 90%, 70% and 50%). For each dewaxing step the sections remained for 4 minutes in the corresponding solution. They were then transferred in methanol supplemented with $H_2O_2$ (30% (v/v)) for 30 minutes and washed twice with PB buffer.

Tissue slices were then microwaved three times in a citrate solution (10mM) at 700W. After 20 minutes of cooling, sections were rinsed several times in water and in washing buffer. To block unspecific binding of anti-pElk antibodies, sections were incubated in blocking buffer (PB buffer supplemented with 0.25% (v/v) TritonX and 5% (v/v) goat serum) for two hours at room temperature. Afterwards, slices were incubated overnight at 4°C with anti-phospho-Elk1 diluted in blocking buffer (Cell Signaling, 9182). Before the addition of biotinylated goat-anti-rabbit IgG (Vectastain elite kit) for two hours at room temperature, sections were washed with washing buffer. Sections were again washed and then treated with a avidin/biotinylated horseradish peroxidase complex reagent, prepared following the instructions of the manufacturer (ABC, Vectastain elite kit). After 30 minutes, slices were washed with water and PB buffer and developed with a solution of diaminobenzidine tetrahydrochloride (Sigma). Oxidation of diaminobenzidine by the peroxidase complex produced a detectable brown color. Therefore phosphorylated Elk1 were stained in brown and detectable with a microsope. Nuclei were counterstained with Hematoxylin.

After washing the section were dehydrated in ethanol solutions with increasing ethanol concentrations (50%, 70%, 90% and 100%), transferred to Rotihistol and embedded in DPX mountant for histology (Sigma).

Heart sections were then photographed with a Leica DM4000B microscope (×5).

| | | |
|---|---|---|
| PB buffer (0.1M) | $Na_2HPO_4$ (1M) | 77.4 ml |
| | $NaH_2PO_4$ (1M) | 22.6 ml |
| | distillated water | ad 1l |
| | pH 7.4 | |
| PB buffer (0.01M) | PB buffer (0.1M) | 10% v/v |
| | distillated water | |
| Washing-buffer | PB buffer (0.1M) supplemented with | |
| | TritonX | 0.25% (v/v) |
| Blocking-buffer | PB buffer (0.1M) supplemented with | |
| | TritonX | 0.25% (v/v) |
| | goat serum | 5% (v/v) |
| Citrate solution | citric acid (0.1M) | 9ml |
| | sodium citrate (0.1M) | 41 ml |
| | distillated water | ad 500 ml |
| Diaminobenzidine tetrahydrochloride | DAB [1 tablet (10mg)] | 0.05% (w/v) |
| | $H_2O_2$ | 0.03% (v/v) |
| | $NiNH_4$-sulfat | 0.04% (v/v) |
| | PB buffer (0.01M) | |

## 5. **Microscope analysis**

Heart sections stained with (H&E) (cf. Methods XI.2) were photographed with a Leica DM4000B microscope (40×0.75). To determine individual cardiomyocyte hypertrophy cross-sectional cell areas were examined with Photoshop (Adobe). Only the cell areas of transversally sectioned cardiomyocytes with a central nucleus were determined by pixel counting. 50 individual cells per animal and 7-9 animals per genotype and group were analyzed.

Heart sections stained with Sirius Red (cf. Methods XI.3) were photographed with a Leica DM4000B microscope (×5) and analyzed with Photoshop (Adobe). To determine interstitial fibrosis a complete transverse heart section was analyzed per animal. Vessels were excluded using the lasso tool of Photoshop. The percentage of fibrosis, i.e. red staining/pixels, was determined by semi-automated image analysis. 7 to 9 animals per genotype and group were included in the analysis.

Fibrosis analyses were performed in a blind-fashion and were analogous for all animals.

# XII. RNA QUANTIFICATION

## 1. RNA extraction from mouse hearts

The RNA from mice ventricles was extracted according to the protocol of RNeasy® Kit (Qiagen). Briefly, hearts were homogenized using an Ultra-Turrax®. The homogenates were incubated for 20 minutes at 55°C and then centrifuged for 5 minutes (4500rpm; RT). Supernatants were supplemented with ethanol (96% (v/v)) and transferred to a RNeasy Midi Spin Columns (Qiagen). After washing, RNA was eluted in fresh RNA-free reaction tubes with RNA-free water and stored in aliquots at -80°C.
To prepare the RNA-free water or DPEC water, 0.1% (v/v) of DEPC were stirred in distillated water for one hour. The solution was then autoclaved

## 2. Transcription from RNA into cDNA

RNA concentration was measured with a NanoDrop 2000c spectrophotometer (Peqlab) at 260 nm. 1µg of RNA was then diluted in RNA-free water (ad 9µl) and reverse transcribed into cDNA using Superscript II reverse transcriptase (Invitrogen).

| | |
|---|---|
| transcription mix | 9 µl RNA dilution |
| | 2 µl oligo dT |
| | 1 µl dNTP (10mM) |

The first step to convert mRNA in cDNA is the formation of the complementary DNA strand of RNA. Oligo dT prime the poly-A 3'tail of the mRNA. For this first strand synthesis the components were transferred in a reaction tube and incubated for 10 minutes at 70°C. The reaction was stopped on ice. Then, reverse transcription was started by addition of 8µl mastermix and incubation for 1 hour at 60°C. The solution was heated at 70°C for 15 minutes to inactivate the reaction. The samples were then diluted in water and stored at -20°C. The new synthetized cDNA could then used as a template for PCR.

mastermix 4 µl enzyme buffer (First Strand, Invitrogen)
2 µl DTT (100 mM)
0.9 µl DEPC water
0.1 µl RNase-inhibitor (RNasin, 40U/µl, Promega)
1 µl Superscript®II Reverse Transcriptase (200U/µl)

## 3. Real-time polymerase chain reaction

The real-time PCR (Higuchi et al, 1993) is a specific form of the normal and commonly used PCR (cf. Methods III.1). While standard PCR gives qualitative results, quantitative results are obtained with real-time PCR. Indeed, during real-time PCR the increase of DNA can be followed at each cycle thanks to the EvaGreen dye. The dye binds to the double strand DNA and its fluorescence is detected each time a new amplicon is formed. The amount of fluorescence is therefore proportional to the amount of DNA and can be quantified.
Reverse transcribed total RNA from mouse hearts were measured in triplicates (3*2.5 µl) on 384 well/plate (BioRad). 10 µl of the following RT-PCR mix were added to each sample. The PCR reaction was performed in a C1000 thermal cycler CFX384™ real-Time System (BioRad) with the CFX Manager Program (BioRad) under the following conditions. The software (CFX Manager Program) recorded constantly the amount of fluorescence.

RT-PCR mix 0.2 µl primer forward (cf. Material VII.2)
0.2 µl primer backward (cf. Material VII.2)
6.25 µl the SsoFast™ EvaGreen® supermix (BioRad)
3.35 µl $H_2O$

| Temperature (°C) | Time | Number of cycle | Step |
|---|---|---|---|
| 98 | 120 | 1 | for denaturation |
| 98<br>56<br>40 | 20<br>20<br>40 | 39 | denaturation<br>annealing<br>elongation |
| 65 | 300 | 1 | end of elongation |

At the beginning of the PCR there is not enough amplicon to detect clearly the fluorescent signal. Then the amount of DNA and thus fluorescence increases in an exponential fashion to reach at the end a plateau. A threshold line is arbitrary determined in this exponential phase, far from the background noise. For each sample the software (CFX Manager Program) measured the cycle number at which the fluorescence crossed the threshold line and this crossing point is called Ct value. More diluted sample cross the threshold line at later Ct value. This Ct value was recorded for each sample. At the end, the results were analyzed with Excel using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

The Ct values of the gene of interest, i.e. ANF (atrial natriuretic factor), were normalized to the Ct values of a housekeeping or reference gene, i.e. GAPDH (Glyceraldehyde 3-phosphate dehydrogenase).

$\Delta Ct_{sample} = Ct_{gene\ of\ interest} - Ct_{housekeeping\ gene}$

GAPDH plays an important role in the glycolysis and is thus expressed in most cells. Since GAPDH is stably expressed in most tissues and cells, it is considered as housekeeping gene (Diez & Simm, 1998). The $\Delta Ct$ of each sample was then compared with the $\Delta Ct$ of the control, i.e. non treated wild-type mice.

$\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{control}$

The fold change $2^{-\Delta\Delta Ct}$ was determined for each group and compared to 1, the ideal value of the control. Since the samples for the gene of interest and the housekeeping gene were measured in triplicates, the quality of the triplicates was determined by calculation of the standard deviation.

Finally, for each primer pairs, a standard curve was plotted to control the efficiency of the PCR.

The EvaGreen dye does not distinguish one double strands DNA from another. The specificity of the fluorescence signal was therefore controlled with a melt curve recorded by the software for each sample.

# XIII. STATISTICAL ANALYSES AND SOFTWARE

Microscope images were recorded with either Leica LAS AF software or Diskuss software and examined with either Photoshop (Adobe) or ImageJ. Nuclear-to-cytosol ratios were analyzed by pixel counting using ImageJ software. Real-time PCR data were normalized in Excel using the $2^{-\Delta\Delta C\,T}$ method.

For statistical analyses GraphPad Software (Prism; version 4.0a) was used. Significance between groups was analyzed by one-way ANOVA followed by Bonferroni test as *post-hoc* test and considered significant with $P<0.05$.

Individual experiments were repeated at least three times. Confocal, histological and physiological analyses were performed in a blind-fashion. Adobe Illustrator was used for graph and figure design.

# RESULTS

## I. β-adrenergic receptors mediate Erk2$^{Thr188}$-phosphorylation

β-adrenergic receptors are known to activate Erk1/2 (Bogoyevitch et al, 1996; Goldsmith & Dhanasekaran, 2007; Heineke & Molkentin, 2006; Zheng et al, 2010). Recently, Lorenz et al. have shown that upon $G_q$-coupled receptor stimulation activated Erk1/2 are involved in a new pathway leading to a third phosphorylation of Erk2 at threonine 188 (Lorenz et al, 2009a; Lorenz et al, 2009b). To determine whether β-adrenergic receptors can also induce Erk2$^{Thr188}$-phosphorylation, human embryonic kidney (HEK293) (Galandrin & Bouvier, 2006; Schmitt & Stork, 2002) cells were transiently transfected with Flag-tagged wild-type Erk2 and $\beta_1$-adrenergic receptors and treated with isoproterenol (5μM), a β-adrenergic receptor agonist (Lohse et al, 2003; Metrich et al, 2008; Osadchii, 2007; Zheng et al, 2010). Cell lysates were separated on SDS-page electrophoresis and analyzed by Western blot. For immunoblot detection of Erk1/2 phosphorylation states, antibodies directed either against the canonical phosphorylation of Erk1/2 at threonine 183 and tyrosine 185 ("TEY motif") [pErk(TEY)] or against Thr188-phosphorylation of Erk1/2 [pErk(T188)] (Lorenz et al, 2009a) were used.

As shown by Lorenz et al., Erk phosphorylation at threonine 188 occurs only when Erk1/2 have been previously phosphorylated at both threonine 183 and tyrosine 185, also called TEY motif. Phosphorylation at the TEY motif is induced by the mitogen-activated protein kinase kinases (Mek1/2), direct activators of Erk1/2 and mirrors canonical Erk1/2 activity (Heineke & Molkentin, 2006; Lorenz et al, 2009a; Lorenz et al, 2009b; May & Hill, 2008; Payne et al, 1991; Seger & Krebs, 1995). Erk1/2 activity was therefore an important element to investigate.

Western blot analyses revealed that 10 minutes of isoproterenol treatment are sufficient to enhance canonical phosphorylation of Erk (Figure 9). Interestingly Erk2$^{Thr188}$-phosphorylation is significantly increased after isoproterenol stimulation in comparison to basal conditions (Figure 9).

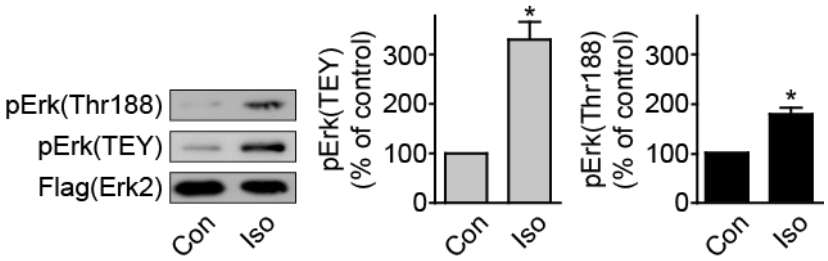



**Figure 9. Activation of overexpressed $\beta_1$-adrenergic receptors mediates Erk2$^{Thr188}$-phosphorylation.** Representative immunoblots of HEK293 cells overexpressing $\beta_1$-adrenergic receptors and Flag-tagged Erk2. Cells were treated without or with isoproterenol (Iso; 5µM) for 10 minutes. Specific antibodies directed against the TEY motif [pErk(TEY)] or Thr188-phosphorylation [pErk(Thr188)] were used for immunoblot analyses and their quantification. *n*=6-9 experiments; *, *P*<0.01.

Erk$^{Thr188}$ phosphorylation was also observed in neonatal rat cardiomyocytes (NRCMs) in response to β-adrenergic receptor activation. NRCMs were isolated (Chlopcikova et al, 2001; Simpson & Savion, 1982) and endogenously expressed β-adrenergic receptors (Lohse et al, 2003) were stimulated with isoproterenol (5µM) for ten minutes. Phenylephrine, which specifically activates endogenous $G_q$-coupled $\alpha_1$-adrenergic receptors in NRCMs (Barki-Harrington et al, 2004; Henaff et al, 2000) and, which has already been shown to induce Erk2$^{Thr188}$-phosphorylation (Lorenz et al, 2009a; Lorenz et al, 2009b) was used as positive control.

Immunoblot analyses showed a significant increase in phosphorylation at the TEY-motif as well as at threonine 188 of Erk after 10 minutes of isoproterenol treatment (Figure 10). The extent of phosphorylation was similar upon $\beta_1$- and $\alpha_1$-adrenergic receptor activation (Figure 10).

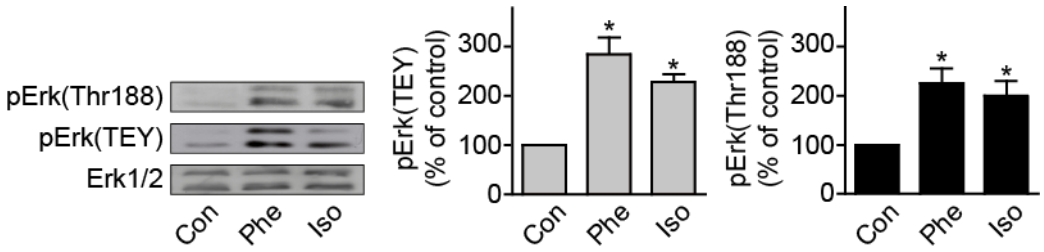



**Figure 10. β-adrenergic receptor activation triggers Erk2$^{Thr188}$-phosphorylation in NRCMs.** Representative immunoblots of NRCMs treated with isoproterenol (Iso; 5μM) or with phenylephrine (Phe; 2μM) for 10 minutes. Specific antibodies directed against the TEY motif of Erk1/2 [pErk(TEY)] or ErkThr188-phosphorylation [pErk(Thr188)] were used for immunoblot analyses and their quantification. *n*=4-6 experiments; *, *P*<0.05.

Same experiments were performed with long-term isoproterenol treatment. Neonatal rat cardiomyocytes were incubated 24 hours without or with isoproterenol (5μM). To avoid degradation of isoproterenol, ascorbic acid (100μM) was added to to the media. AngiotensinII (100nM) was used as positive control since angiotensinII was shown to mediate Erk2$^{Thr188}$-phosphorylation in adult cardiac myocytes (Lorenz et al, 2009a).

Long-term isoproterenol treatment induced an increase of phosphorylation at TEY. Erk2$^{Thr188}$-phosphorylation was also under these conditions significantly increased by either isoproterenol or angiotensinII treatment (Figure 11).

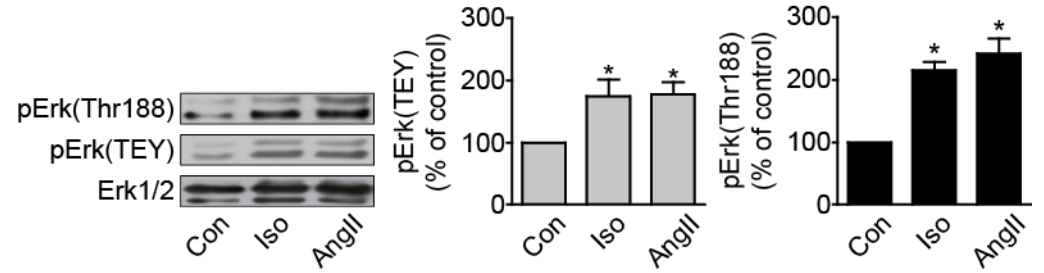



**Figure 11. Erk2$^{Thr188}$-phosphorylation is mediated by β-adrenergic receptors in NRCMs.** Representative immunoblots of NRCMs treated with isoproterenol (Iso; 5μM) or with angiotensinII (AngII; 100nM) for 24 hours. Specific antibodies directed against the TEY motif [pErk(TEY)] or Thr188-phosphorylation [pErk(Thr188)] were used for immunoblot analyses and their quantification. *n*=6-8 experiments; *, *P*<0.05.

Western blot analyses were also performed with lysates from isolated adult cardiomyocytes (CMs). Direct after isolation adult CMs were treated without or with isoproterenol (5µM) for six minutes. In accordance with the results observed in NRCMs, activation of endogenous β-adrenergic receptors triggers phosphorylation at the TEY-motif and Erk2$^{Thr188}$-phosphorylation. Indeed both phosphorylations increased significantly in response to isoproterenol activation (Figure 12).

Taken together these data show that β-adrenergic receptor activation induces Erk2$^{Thr188}$-phosphorylation *in vitro*.

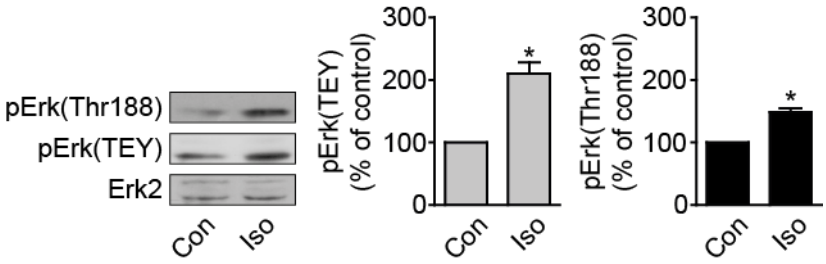



**Figure 12. Activation of β-adrenergic receptors in adult cardiomyocytes induces Erk2$^{Thr188}$-phosphorylation.** Representative immunoblots of adult cardiomyocytes treated without or with isoproterenol (Iso; 5µM) for 6 minutes. Specific antibodies directed against the TEY motif [pErk(TEY)] or Thr188-phosphorylation [pErk(Thr188)] were used for immunoblot analyses and their quantification. *n*=3-5 experiments; *, *P*<0.05.

Phosphorylation at the TEY-motif of Erk1/2 and Erk$^{Thr188}$-phosphorylation shown after β-AR activation *in vitro* were also observed *in vivo*. Transgenic mice (FVB/N) with cardiac overexpression of wild-type Erk2 were exposed to short-term dobutamine treatment (220 ng/g body weight/6 minutes) (Casella et al, 2011; Hoit et al, 1997). After treatment, left ventricles were collected, homogenized and lysed. Erk phosphorylations were detected by Western blot analyses. Left ventricles of non-treated transgenic mice served as controls.

In line with the *in vitro* data, phosphorylation at the TEY-motif of Erk1/2 and Erk$^{Thr188}$-phosphorylation were significantly increased after dobutamine treatment (Figure 13).

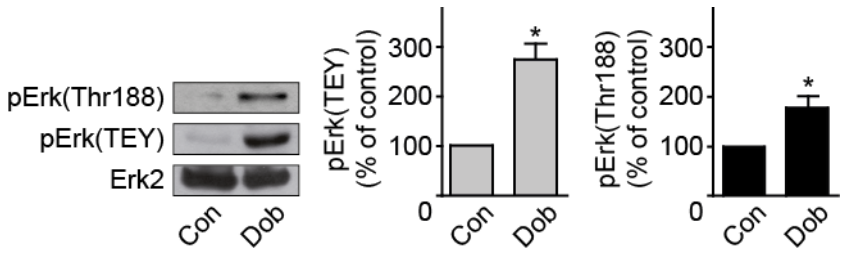



**Figure 13. β-adrenergic receptors activation induces Erk2$^{Thr188}$-phosphorylation *in vivo*.** Representative immunoblots of heart lysates from transgenic mice with a cardiac overexpression of Erk2$^{T188T}$ treated without or with dobutamine (Dob; infusion via *V. jugularis*; 220 ng/g body weight/6 minutes). Specific antibodies directed against the TEY motif [pErk(TEY)] or Thr188-phosphorylation [pErk(Thr188)] were used for immunoblot analyses and their quantification. *n*=7-8 experiments; *, *P*<0.05.

Interestingly, same effects were obtained upon sustained activation of β-adrenergic receptors. Osmotic mini-pump containing isoproterenol (30mg/kg/day) were implanted subcutaneously in wild-type mice (FVB/N). 14 days later, left ventricles were collected, homogenized and analyzed by Western blots. Erk phosphorylations were detected by specific antibodies directed against the TEY-motif and Thr188-phosphorylation.

While phosphorylation canonical Erk1/2 phosphorylation was not increased, Erk2$^{Thr188}$-phosphorylation was increased in mice treated with isoproterenol in contrast to non-treated control mice (Figure 14).

Taken together, *in vitro* and *in vivo* data show that β-adrenergic receptors induce Erk$^{Thr188}$-phosphorylation upon isoproterenol activation.

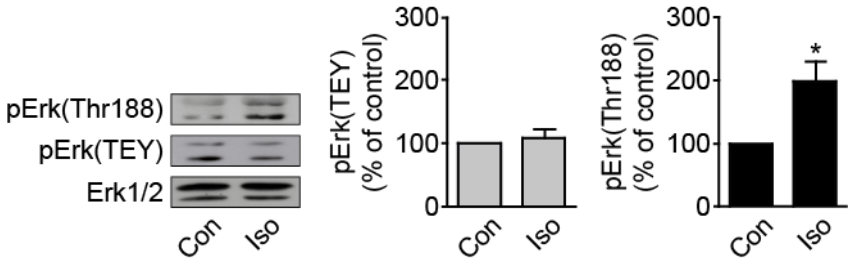



**Figure 14. Sustain activation of β-adrenergic receptors with isoproterenol mediates Erk2$^{Thr188}$-phosphorylation *in vivo*.** Representative immunoblots of heart lysates from wild type FVB/N mice treated with isoproterenol (Iso; 30mg/kg/day) via osmotic mini-pump implanted subcutaneously for 2 weeks. Non-treated mice served as control. Specific antibodies directed against the TEY motif [pErk(TEY)] or Thr188-phosphorylation [pErk(Thr188)] were used for immunoblot analyses and their quantification. *n*=4 experiments; *, *P*<0.05.

## II. β-adrenergic receptor activation induces cardiomyocyte hypertrophy via Erk2$^{Thr188}$-phosphorylation *in vitro*

The role of Erk1/2 in β-adrenergic receptor mediated hypertrophy was investigated in isolated neonatal rat cardiomyocytes (NRCMs). These cells are widely used in cardiovascular research, since they are easier to isolate and have a better viability in cell culture than adult cardiomyocytes. Although NRCMs are separated from one another and lose their rectangular shape, they maintain their contractile ability upon stimulation. Moreover, they can be transduced by different technics (Adams & Brown, 2001) and have been already shown to undergo hypertrophy in response to different agonists, such as catecholamine (Chlopcikova et al, 2001; Woodcock & Matkovich, 2005; Zierhut & Zimmer, 1989). For these reasons, the following experiments were performed in isolated neonatal rat cardiomyocytes. α-actinin staining, to visualize cardiomyocytes and DAPI staining to visualize cell nuclei were used to analyze the purity of the NRCM preparation. Purity was then identified by confocal microscopy. More than 95% of the isolated cells were NRCMs (Figure 15). Less than 5% of the detected cell nuclei were only DAPI but not α-actinin positive. And to prevent non-myocyte proliferation, NRCMs were cultured in serum-free media supplemented with bromodeoxyuridine (BrdU) (Simpson & Savion, 1982).

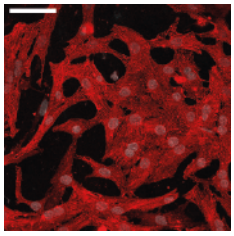


**Figure 15. Purity of neonatal rat cardiomyocyte preparation was more than 95%.** Representative confocal picture of NRCMs. 48 hours after isolation NRCMs were stained with α-actinin antibodies and nuclei were stained with DAPI. Scale bar represents 50 µm.

To determine the involvement of Erk1/2 in cardiac hypertrophy upon β-adrenergic receptor activation, NRCMs were pretreated with PD98059 (30µM), a potent and selective inhibitor of the upstream kinases of Erk1/2, Mek1/2 (Sugden & Clerk, 1998; Teos et al, 2008; Zheng et al, 2010). After 90 minutes of PD98059

treatment, NRCMs were incubated for 30 hours without or with isoproterenol (5µM) in presence of ascorbic acid (100µM) and tritiated [$^3$H]-isoleucine (1µCi/ml). Tritiated [$^3$H]-isoleucine is incorporated in newly synthetized protein and can monitor protein synthesis in response to hypertrophic signals. Cardiomyocyte hypertrophy was therefore monitored by [$^3$H]-isoleucine incorporation (Buitrago et al, 2005; Lorenz et al, 2009a).

Interestingly, β-adrenergic receptor mediated hypertrophy was inhibited by Mek1/2 inhibition. While isoproterenol in absence of the Mek1/2 inhibitor PD98059 induced a significant increase in cardiomyocyte hypertrophy, the hypertrophic response was significantly attenuated in the presence of PD98059 (Figure 16).

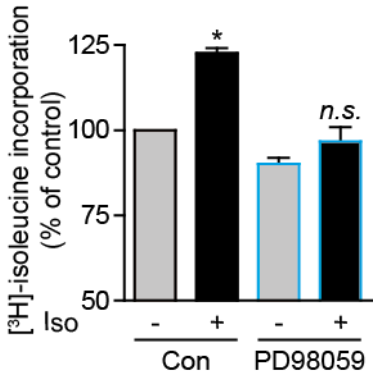



**Figure 16. PD98059 inhibits isoproterenol induced cardiomyocyte hypertrophy.** [$^3$H]-isoleucine incorporation in NRCMs. Cells were pretreated with PD98059 (30µM; 30 minutes) and stimulated without or with isoproterenol (Iso; 5µM) in presence of ascorbic acid (100µM) for 24 hours. *n*=7 experiments; *, *P*<0.001 *versus* all other conditions; *n.s. versus* unstimulated controls.

PD98059 efficiency on Erk1/2 inhibition was controlled by Western blot analysis (Figure 17). Erk1/2 activity, mirrored by phosphorylation at the TEY-motif, was completely inhibited in NRCMs pretreated with PD98059. PD98059 also prevented Erk2$^{Thr188}$-phosphorylation (Figure 17). In the absence of PD98059, isoproterenol showed a significant increase in both phosphorylations of Erk1/2.

Altogether these results prove the importance of Erk1/2 in isoproterenol-induced cardiomyocyte hypertrophy. The involvement of Erk2$^{Thr188}$-phosphorylation in this

hypertrophic signaling pathway was therefore investigated in the following experiments.

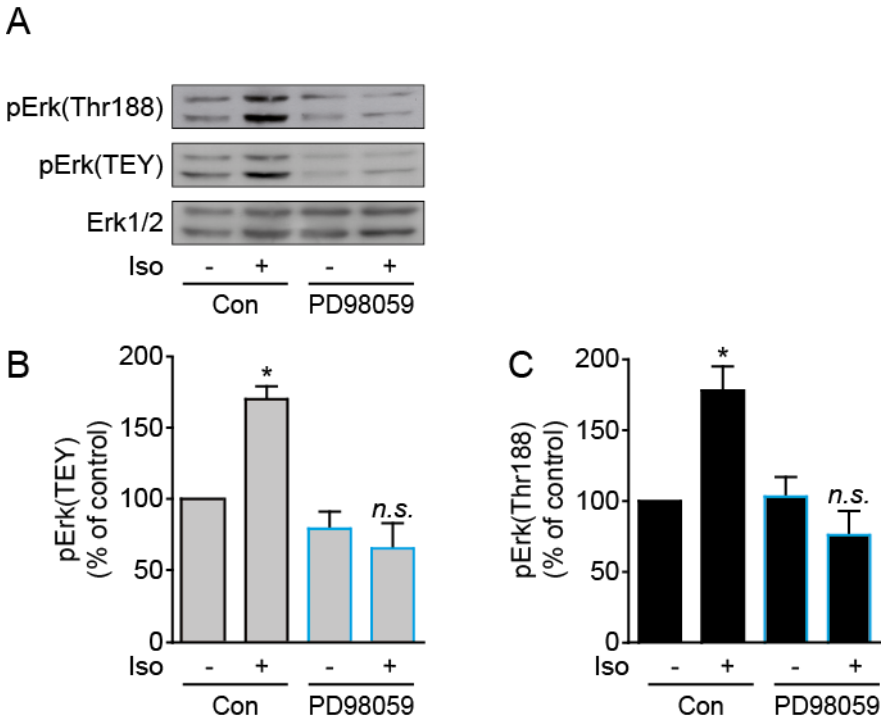



**Figure 17. PD98059 prevents β-adrenergic receptor induced Erk2$^{Thr188}$-phosphorylation.** Representative immunoblots of NRCMs pretreated with PD98059 (30μM; 30 minutes) and stimulated without or with isoproterenol (Iso; 5μM) for 24 hours. Specific antibodies directed against the TEY motif [pErk(TEY)] or Thr188-phosphorylation [pErk(Thr188)] of ERK1/2 were used for immunoblot analyses and their quantification. *n*=6 experiments; *, *P*<0.05 *versus* all other conditions; *n.s. versus* unstimulated controls.

To determine whether Erk2$^{Thr188}$-phosphorylation is also involved in β-adrenergic receptors induced cardiomyocyte hypertrophy, three different adenoviruses were generated. These adenoviruses encoded either for N-terminally Flag-tagged wild-type Erk2 (T188T) or for two phosphorylation-deficient mutants Flag-Erk2$^{T188S}$ (T188S) and Flag-Erk2$^{T188A}$ (T188A). In T188S and T188A mutants, threonine 188 was exchanged by a serine or an alanine, respectively, to prevent phosphorylation of Erk2 at Thr188. Lorenz et al. have previously described these two mutants, T188S and T188A, and justified their disability to phosphorylate by phosphopeptide mapping analysis (Lorenz et al, 2009a). 48 hours after cell

isolation, NRCMs were transformed using these adenoviruses (Figure 18) (Bueno et al, 2000; Merkle et al, 2007).

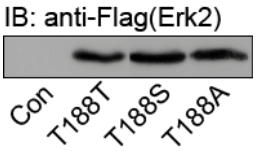



**Figure 18. Wild-type Flag-Erk2 (T188T) and the phosphorylation-deficient mutants Flag-Erk2T$^{188S}$ (T188S) and Flag-Erk2$^{T188A}$ (T188A) were equally expressed in NRMCs.** Immunoblot analysis using specific antibodies directed against the N-terminal Flag-epitope. NRCMs were transformed 48 hours after cell isolation.

The functionality of the three adenoviruses, wild-type Flag-Erk2 (T188T), Flag-Erk2$^{T188S}$ (T188S) and Flag-Erk2$^{T188A}$ (T188A), mirrored by phosphorylation at the TEY-motif was controlled by Western blot analysis (Figure 19). Indeed the mutation in T188S and T188A should not impede the phosphorylation at the TEY-motif, which is induced by MEK activity and reflects Erk1/2 activity.

Western blot analysis revealed that Erk1/2 activity is similar for T188T and the phosphorylation deficient mutants T188S and T188A upon β-adrenergic receptors stimulation (Figure 19).

T188T, T188S and T188A were used in the following experiments and the results obtained were therefore comparable

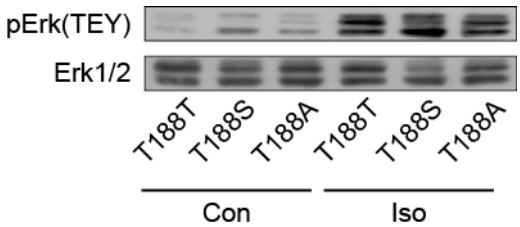



**Figure 19. T188T, T188S and T188A are similarly activated by β-adrenergic receptors.** Immunoblot analysis using specific antibodies directed against the TEY motif [pErk(TEY)]. NRCMs transfected with wild-type Flag-Erk2 (T188T) or the phosphorylation-deficient mutants Flag-Erk2$^{T188S}$ or Flag-Erk2$^{T188A}$ (respectively T188S or T188A) were treated without or with isoproterenol (Iso; 5μM) for 10 minutes.

To determine whether overexpression of Erk2 wild-type itself has an influence on β-adrenergically mediated hypertrophy, mock-transfected cells (GFP) were compared to wild-type Erk2 overexpressing cardiomyocytes (T188T). Transfected neonatal rat cardiomyocytes were treated without or with isoleucine (5µM) in presence of ascorbic acid (100µM) and [$^3$H]-isoleucine (1µCi/ml). After 30 hours, NRCMs were washed with PBS, proteins were precipitated in TCA and then solubilized under basic conditions. Cardiomyocyte hypertrophy was then determined by quantification of incorporated, tritiated isoleucine.
After isoproterenol treatment a similar increase in hypertrophy was observed in mock-transfected and T188T overexpressing cells (Figure 20).

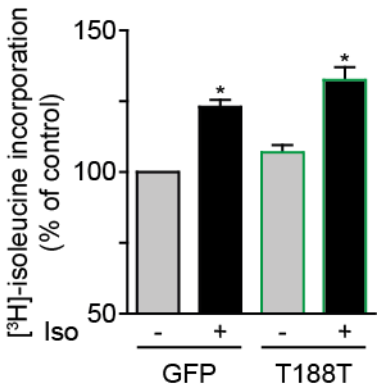



**Figure 20. Overexpression of wild-type Erk2 in NRCMs does not influence the extent of isoproterenol induced hypertrophy.** [$^3$H]-isoleucine incorporation assay and in mock-transfected (GFP) or wild-type Erk2 (T188T) overexpressing NRCMs treated without or with isoproterenol (Iso; 5µM; 30 hours) in presence of ascorbic acid (100µM). *n*=5 experiments; *, *P*<0.01 *versus* unstimulated controls.

An involvement of Erk2$^{Thr188}$-phosphorylation in β-adrenergic receptor mediated hypertrophic response was detected in [$^3$H]-isoleucine incorporation assays. Since Erk2$^{Thr188}$-phosphorylation was known to enhance cardiac hypertrophy in response to Gq-coupled receptor activation (Lorenz et al, 2009a), phenylephrine, a specific agonist of $\alpha_1$-adrenergic receptors, was used as positive control (Barki-Harrington et al, 2004; Kehat et al, 2011; Lorenz et al, 2009a).
Wild-type Erk2 (T188T) and the phosphorylation-deficient mutants Erk2$^{T188S}$ (T188S) or Erk2$^{T188A}$ (T188A) overexpressed NRCMs were incubated without or with phenylephrine (2µM) and [$^3$H]-isoleucine (1µCi/ml). After 30 hours, tritiated isoleucine was quantified.

While overexpression of wild-type Erk2 enhanced significantly the hypertrophic effect of phenylephrine, both Erk2$^{T188S}$ and Erk2$^{T188A}$ attenuated it (Figure 21) (Lorenz et al, 2009a).

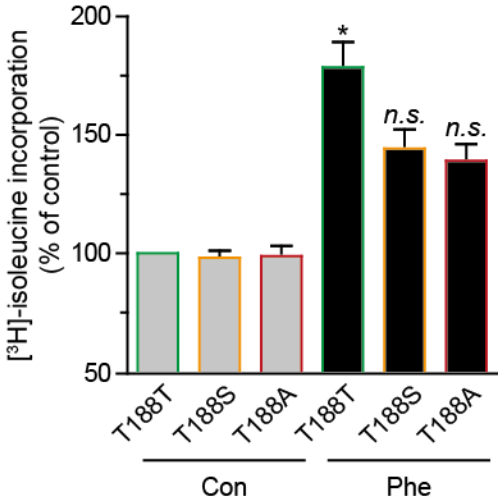



**Figure 21. Erk$^{Thr188}$-phosphorylation mediates phenylephrine induced cardiomyocyte hypertrophy in NRCMs.** [$^3$H]-isoleucine incorporation assay in NRCMs transfected with wild-type Erk2 (T188T), Erk2$^{T188S}$ (T188S) or ERk2$^{T188A}$ (T188A). Cells were treated without or with phenylephrine (Phe; 2µM) for 30 hours in presence of ascorbic acid (100µM) and [3H]-isoleucine (1µCi/ml). *n*=9-13 experiments; *, *P*<0.01 *versus* all other conditions; *n.s. versus* unstimulated controls.

Following the same protocol, NRCMs transfected with either wild-type Erk2 (T188T) or the phosphorylation-deficient mutants Erk2$^{T188S}$ (T188S) and Erk2$^{T188A}$ (T188A) were exposed to isoproterenol (5µM) in cell culture medium supplemented with ascorbic acid (100µM) and [$^3$H]-isoleucine (1µCi). After 30 hours, [$^3$H]-isoleucine incorporation revealed that cardiomyocytes overexpressing wild-type Erk2 (T188T) developed notable hypertrophy in response to isoproterenol in comparison to unstimulated cells (Figure 22). Interestingly, the hypertrophic effect of isoproterenol was also significantly diminished in NRCMs overexpressing Erk2$^{T188S}$ (T188S) and Erk2$^{T188A}$ (T188A) (Figure 22).

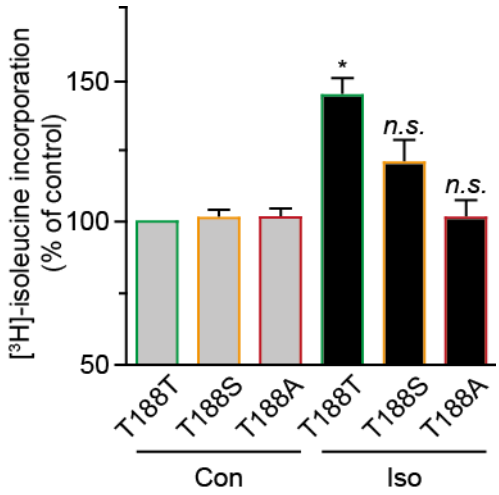



**Figure 22. Erk$^{Thr188}$-phosphorylation enhances isoproterenol-induced cardiomyocyte hypertrophy.** [$^3$H]-isoleucine incorporation assay in NRCMs transfected with wild-type Erk2 (T188T), Erk2$^{T188S}$ (T188S) or ERk2$^{T188A}$ (T188A). Cells were treated without or with isoproterenol (Iso; 5µM) for 30 hours in presence of ascorbic acid (100µM) and [$^3$H]-isoleucine (1µCi/ml). *n*=11-19 experiments; *, *P*<0.05 *versus* all other conditions; *n.s. versus* unstimulated controls.

In addition to [$^3$H]-isoleucine assays, cell size measurements were performed. These essays are also commonly used to analyze cardiomyocyte hypertrophy (Buitrago et al, 2005; Dorn et al, 2003; Metrich et al, 2008; Morel et al, 2005). As in the previous experiments, isolated NRCMs were transfected with wild-type Erk2 (T188T), Erk2$^{T188S}$ (T188S) or Erk2$^{T188A}$ (T188A). After 24 hours treatment without or with isoproterenol (5µM), actin filaments of cardiomyocytes were stained with phalloidin (alexa fluor 488) and nuclei were stained with DAPI. Cells were analyzed using a confocal microscope.
In line with the [$^3$H]-isoleucine incorporation assays (Figure 22) under these experimental conditions, overexpression of wild-type Erk2 did not influence the hypertrophic effect of isoproterenol. Indeed mock-transfected and Erk2 overexpressed NRCMs enlarged similarly after isoproterenol treatment (Figure 23).

Interestingly, cardiomyocytes overexpressing phosphorylation-deficient Erk2 mutants, Erk2$^{T188S}$ (T188S) or Erk2$^{T188A}$ (T188A), showed a significantly reduced hypertrophic response to β-adrenergic receptor activation regarding the cell size compared to cardiomyocytes overexpressing wild-type Erk2 (T188T) (Figures 24 and 25).

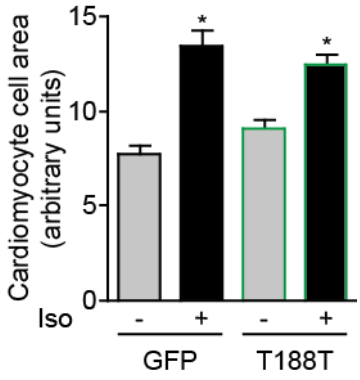



**Figure 23. Overexpression of wild-type Erk2 does not enhance the effects of isoproterenol on cardiomyocyte hypertrophy.** Cell area measurements in mock-transfected (GFP) or wild-type Erk2 (T188T) overexpressing NRCMs treated without or with isoproterenol (Iso; 5µM; 24 hours) in presence of ascorbic acid (100µM). n=10-20 cells per group; *, *P*<0.01 *versus* unstimulated controls.

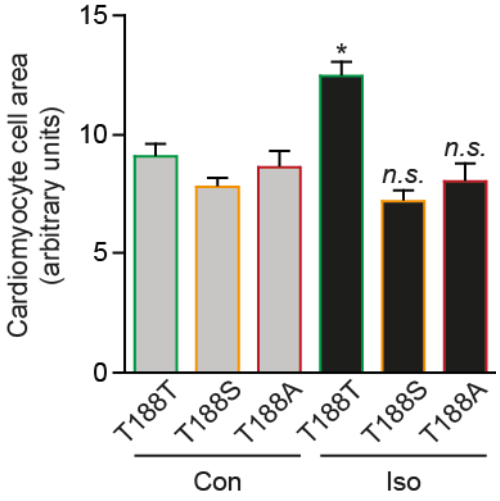



**Figure 24. Phosphorylation deficient mutants Erk2$^{T188S}$ (T188S) and Erk2$^{T188A}$ (T188A) attenuate β-adrenergically induced increase in cell size.** Alexa-fluor-488 phalloidin staining of NRCMs transfected with Erk2 (T188T), Erk2$^{T188S}$ (T188S) or Erk2$^{T188A}$ (T188A). Cells were treated without or with isoproterenol (Iso; 5µM) for 24h, stained with phalloidin and cell areas were quantified. *n*=14-20 cells per group; *, *P*<0.01 *versus* all other conditions; *n.s. versus* unstimulated controls.

Hypertrophy in cardiomyocytes is also associated with up-regulation of actin synthesis and sarcomere reorganization (Aoki et al, 1998; Dorn et al, 2003; Woodcock & Matkovich, 2005). Representative pictures of phalloidin stained NRCMs transfected with wild-type Erk2 (T188T) or Erk2 mutants, which are phosphorylation-deficient for $Erk2^{Thr188}$-phosphorylation showed that the cell size increased and that the sarcomere indeed reorganized in wild-type Erk2 overexpressing cardiomyocytes (T188T). In comparison loss of threonine 188 phosphorylation, mirrored by $Erk2^{T188S}$ (T188S) and $Erk2^{T188A}$ (T188A) mutants, inhibited cytoskeleton reorganization of NRCMs (Figure 25).

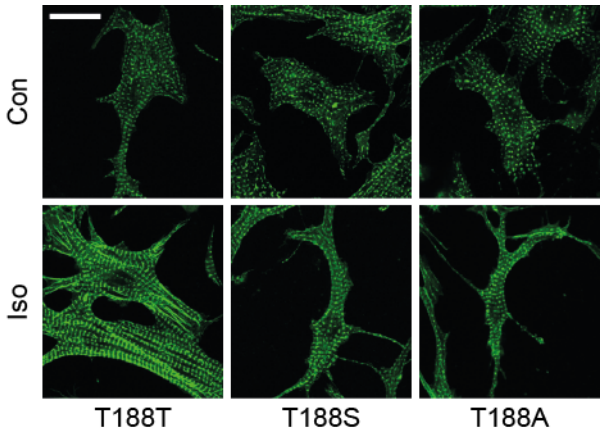



**Figure 25. $Erk2^{Thr188}$-phosphorylation increased cardiomyocyte size and induced actin reorganization.** Representative confocal images of NRCMs transfected with wild-type Erk2 (T188T), $Erk2^{T188S}$ (T188S) or $Erk2^{T188A}$ (T188A). Cells were treated without or with isoproterenol (Iso; 5µM) for 24 hours and then stained with Alexa-fluor-488 phalloidin. Scale bar represents 20µm.

Cell size measurements were also performed in isolated adult cardiomyocytes (CMs). Adult CMs are more difficult to keep in culture than NRCMs and laminin is required to keep them attached to the culture dish. Moreover during isolation CMS detach from each other and their membrane become partially permeable. They need therefore to be kept in $Ca^{2+}$-medium (Woodcock & Matkovich, 2005).
Two hours after cell isolation, CMs were transfected either with N-terminally YFP-tagged wild-type Erk2 (T188T) or the phosphorylation deficient YFP-tagged $Erk2^{T188A}$ (T188A) mutant. Mock-transfcted cells served as additional controls. Cell areas were measured before and after isoproterenol exposition.

In line with NRCM results, overexpression of Erk2$^{T188A}$ impedes the hypertrophic response of isoproterenol unlike endogenous or overexpressed wild-type Erk2. Cardiomyocytes overexpressing wild-type Erk2 develop hypertrophy in a similar extent than mock-transfected cells upon β-adrenergic receptor stimulation (Figure 26).

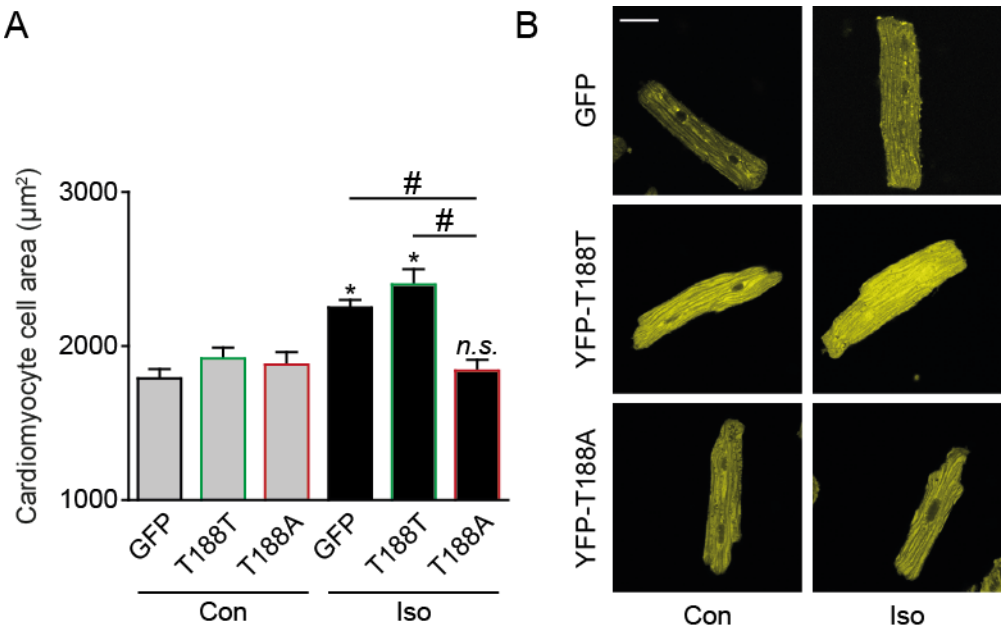



**Figure 26. Overexpression of the phosphorylation deficient mutant YFP-Erk2$^{T188A}$ reduces isoproterenol mediated increase in adult cardiomyocyte area.** (**A**) Cell area measurements and (**B**) representative confocal pictures of individual cardiomyocyte. CMs were transfected with YFP-tagged wild-type Erk2 (T188T) or YFP-Erk2$^{T188A}$ (T188A) and incubated without or with isoproterenol (Iso; 5µM) for twelve hours. Cell areas were quantified in absence or presence of isoproterenol. Mock-transfected cells (GFP) served as additional controls. *n*=51-52 cells per animal; 5 animals per group; *, *P*<0.05 *versus* unstimulated controls; #, *P*<0.01; *n.s. versus* unstimulated controls. The scale bar represents 25µm.

Altogether, these results confirm the involvement of Erk2$^{Thr188}$-phosphorylation in β-adrenergic receptor induced cardiomyocyte hypertrophy *in vitro*. Mutants phosphorylation deficient for Erk2$^{Thr188}$-phosphorylation, Erk2$^{T188S}$ and Erk2$^{T188A}$, significantly attenuate the hypertrophic response of NRCMs and adult CMs to isoproterenol treatment.

## III. β-adrenergic receptor induced cardiac hypertrophy is mediated by Erk2$^{Thr188}$-phosphorylation *in vivo*

The involvement of Erk2$^{Thr188}$-phosphorylation in β-adrenergic receptors induced hypertrophy was investigated *in vivo* to support and confirm the conclusions obtained *in vitro***.** For this study, transgenic mice with cardiac overexpression of either wild-type Erk2 (T188T) or the phosphorylation deficient mutant Erk$^{T188S}$ (T188S) were used (Buitrago et al, 2005; Lorenz et al, 2009a). In addition to transgenic wild-type Erk2 mice, FVB/N wild-type mice served as control.
Erk2$^{T188S}$ and Erk$^{T188A}$, the two phosphorylation deficient mutants of Erk2 showed no significant difference between them after isoproterenol activation *in vitro* (Figures 22, 24 and 25). Therefore only Erk2$^{T188S}$ overexpressing transgenic mice were generated.

To analyze whether Erk2$^{Thr188}$-phosphorylation is induced by β-adrenergic receptor activation *in vivo* and its role *in vivo*, β-adrenergic receptors in mice were stimulated by implantation of osmotic mini-pumps containing isoproterenol (30mg/kg/day). The mini-pumps were implanted subcutaneously for two weeks. Changes in morphology and cardiac function were recorded before and after isoproterenol treatment by histological and echocardiographic analyses. The different wall thicknesses were measured from two-dimensional M-mode images in the short axis view at the proximal level of papillary muscle. VisualSonics Cardiac measurement Software was used to compute the changes and movements of the myocardium.

No difference in morphology and cardiac function was detected under basal conditions between wild-type FVB/N mice and transgenic Erk2 wild-type or T188S mice (Figure 27-36). However after 14 days of isoproterenol treatment, both inter-ventricular septum (IVS) and left ventricular posterior wall (LVPW) thicknesses increased significantly in wild-type mice and wild-type Erk2 overexpressing mice (T188T). Interestingly, wall thicknesses were not increased by isoproterenol in Erk2$^{T188S}$ (T188S) transgenic mice (Figure 27).

Isoproterenol treatment induced a gain in the mass of left ventricles (left ventricle to tibia length) and hearts (ventricle per tibia length) in all genotypes. However, these effects were less pronounced in Erk2$^{T188S}$ transgenic mice in comparison to wild-type or wild-type Erk2 overexpressing mice (Figure 28).

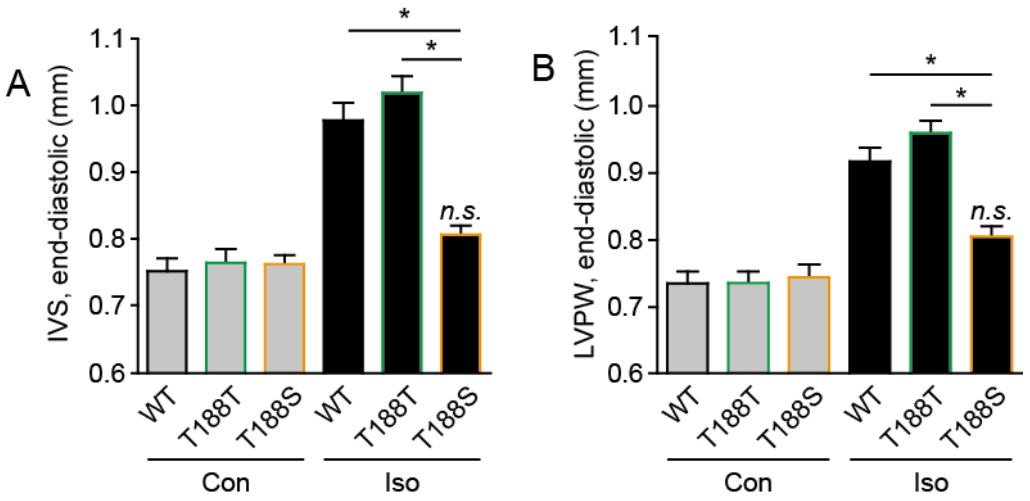



**Figure 27. Erk2$^{Thr188}$-phosphorylation triggers isoproterenol induced cardiac hypertrophy *in vivo*.** Echocardiographic analyses of wild-type and transgenic mice overexpressing either wild-type Erk2 (T188T) or the phosphorylation deficient mutant Erk2$^{T188S}$ (T188S) before and after implantation of osmotic mini-pumps containing isoproterenol (Iso; 30mg/kg/day) for 14 days. End-diastolic measurements of (**A**) intraventricular septum (IVS) and (**B**) left ventricular posterior wall thickness. *n*=5-14 animals per groups; *, *P*<0.01; *n.s. versus* unstimulated controls.

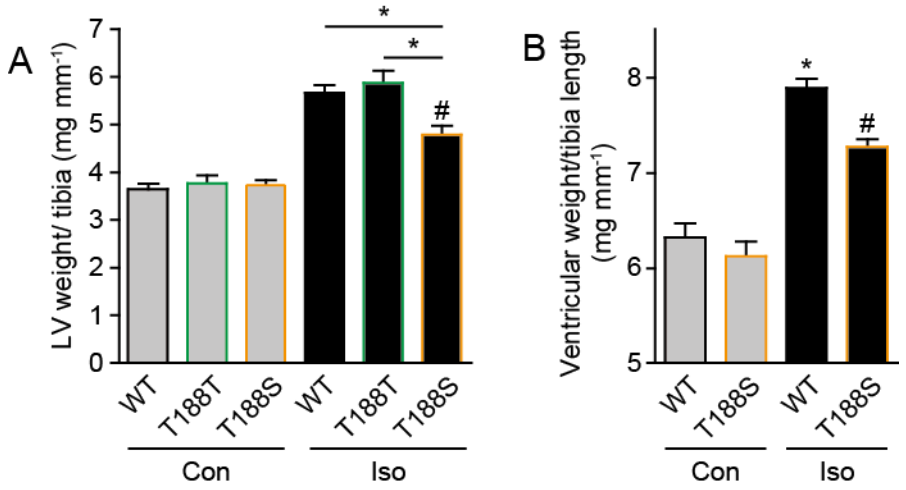



**Figure 25. The increase in the mass of left ventricles and hearts recorded after isoproterenol treatment is enhanced by Erk2$^{Thr188}$-phosphorylation.** Ratios of (A) left ventricular weight to tibia length measured by echocardiography analyses and tibia length and of (B) ventricular weight to tibia length of wild-type mice or transgenic mice either overexpressing wild-type Erk2 (T188T) or the phosphorylation deficient mutant Erk$^{T188S}$ (T188S) as indicated before and after isoproterenol (Iso; 30mg/kg/day) treatment. *n*=5-14 animals per groups; *, *P*<0.01 as indicated or *versus* all other conditions; #, *P*<0.05 *versus* unstimulated controls.

However, two weeks of isoproterenol treatment were not sufficient to reveal any signs of heart failure in mice of the FVB/N genetic background. Left ventricular contractility and function was evaluated by analysis of fractional shortening and ejection. Values for fractional shortening (FS) and ejection fraction (EF) was similar within all genotypes. Isoproterenol treated mice showed even increased values for FS and EF, i.e. did not show any signs of heart failure (Figure 29). Measurements of left ventricular diameters (LVID) showed no significant dilation of left ventricles in wild-type mice or in overexpressed Erk2 and Erk2$^{T188S}$ transgenic mice (Figure 30).

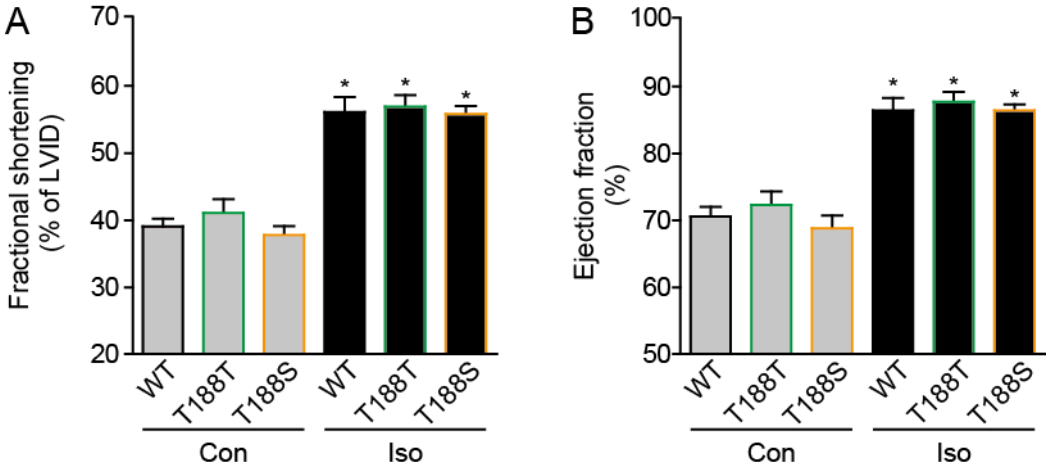



**Figure 29. Two weeks of isoproterenol treatment were not sufficient to induce heart failure in this mouse model.** Echocardiographic analyses of wild-type mice or transgenic mice either overexpressing wild-type Erk2 (T188T) or the phosphorylation deficient mutant $Erk^{T188S}$ (T188S) before and after isoproterenol (Iso; 30mg/kg/day) treatment. Assessment of (**A**) fractional shortening and (**B**) ejection fraction. *n*=5-14 animals per groups; *, *P*<0.01 *versus* unstimulated controls.

Heart rates between genotypes were indistinguishable under basal conditions and after isoproterenol treatment. Heart rate in isoproterenol treated mice, however, was significantly increased (Figure 30).

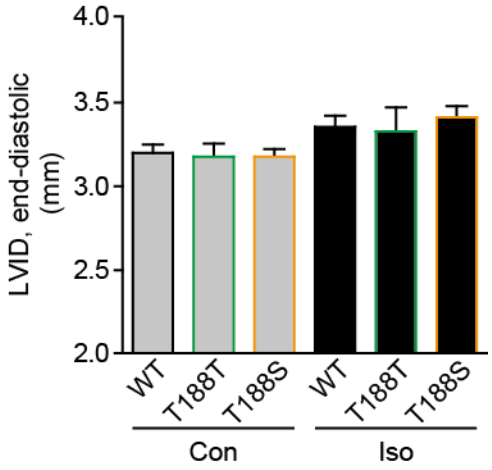



**Figure 30. No left ventricular dilatation was observed after two weeks of isoproterenol treatment in this mouse model.** Echocardiographic analyses of wild-type mice or transgenic mice either overexpressing wild-type Erk2 (T188T) or the phosphorylation deficient mutant Erk$^{T188S}$ (T188S) before and after isoproterenol (Iso; 30mg/kg/day) treatment. End-diastolic measurements of left ventricular interior diameter (LVID). *n*=5-14 animals per groups.

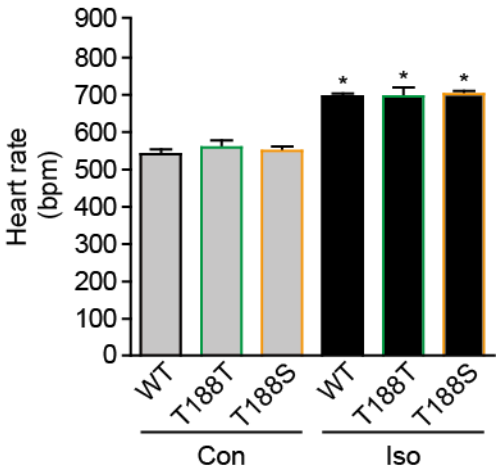



**Figure 31. Erk2$^{Thr188}$-phosphorylation has no influence on isoproterenol increased heart rates.** Assessment of heart rates by echocardiographic analyses of wild-type mice or transgenic mice either overexpressing wild-type Erk2 (T188T) or the phosphorylation deficient mutant Erk$^{T188S}$ (T188S) before and after isoproterenol (Iso; 30mg/kg/day) treatment. *n*=5-14 animals per groups; *, P<0.01 *versus* unstimulated controls.

To confirm that the increase in wall thickness, which was observed by echocardiographic measurements, was due to an increase in cardiomyocyte size, the cardiomyocyte area was analyzed in histological H&E stained sections under basal conditions and after two weeks of isoproterenol treatment.

The result quantification revealed a significant increase in myocyte area of wild-type mice and overexpressing wild-type Erk2 transgenic mice (T188T) upon isoproterenol exposition compared to myocytes of the respective non-treated animals.

Interestingly, cardiomyocyte areas of Erk2$^{T188S}$ (T188S) transgenic mice, which were comparable with the areas determined in wild-type mice or wild-type Erk2 overexpressing mice under basal conditions, did not increase in response to isoproterenol (Figure 32).

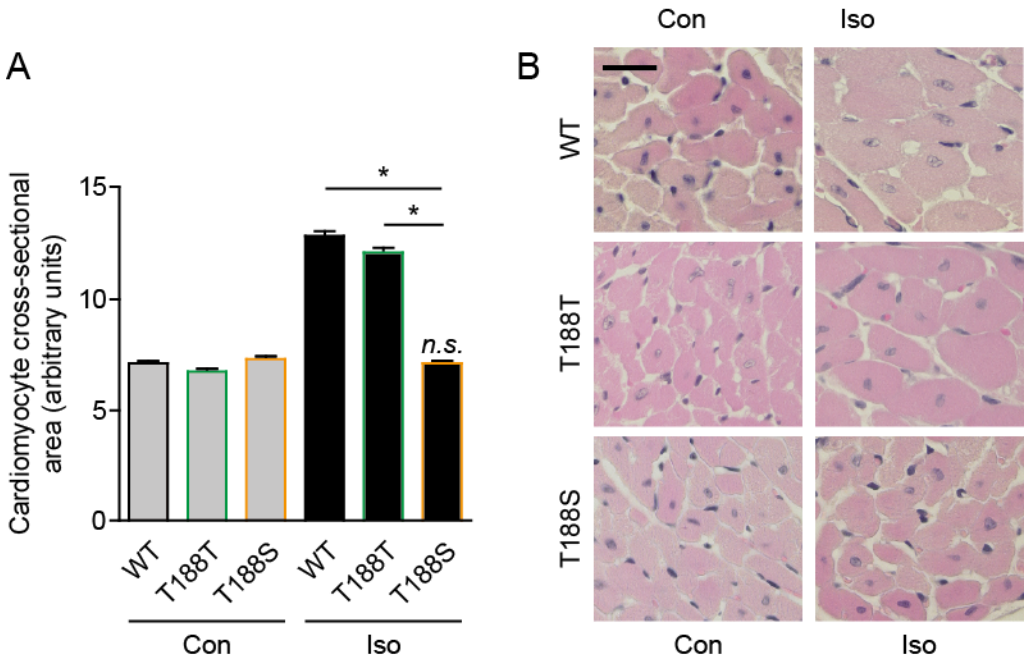



**Figure 32. Thr188-phosphorylation of Erk2 induces an increase in cardiomyocyte area upon β-adrenergic receptor activation.** Histological sections of ventricular myocardium of wild-type mice or transgenic mice overexpressing either wild-type Erk2 (T188T) or the phosphorylation deficient mutant Erk2$^{T188S}$ (T188S) before and after isoproterenol treatment (Iso; 30mg/kg/day). (**A**) Quantification of individual cardiomyocyte cross-sectional areas and (**B**) representative heart sections stained with H&E. Scale bar represents 25µm. *n*=50 cells per animals, 5-8 animals per groups; *, *P*<0.001 as indicated; *n.s. versus* unstimulated controls.

Similar results were obtained in isolated adult cardiomyocytes (Figure 33). CMs isolated from wild-type mice and Erk2$^{T188S}$ transgenic mice were exposed to isoproterenol for twelve hours. Cells were observed under a microscope and cell areas were quantified before and after isoproterenol exposition for each genotype, wild type and Erk2$^{T188S}$.

While under basal conditions wild type and Erk2$^{T188S}$ cardiomyocytes show similar area, only CMs from wild-type mice enlarge significantly in response to isoproterenol. Overexpression of Erk2$^{T188S}$ inhibits the β-adrenergic receptor mediated cell size increase (Figure 33).

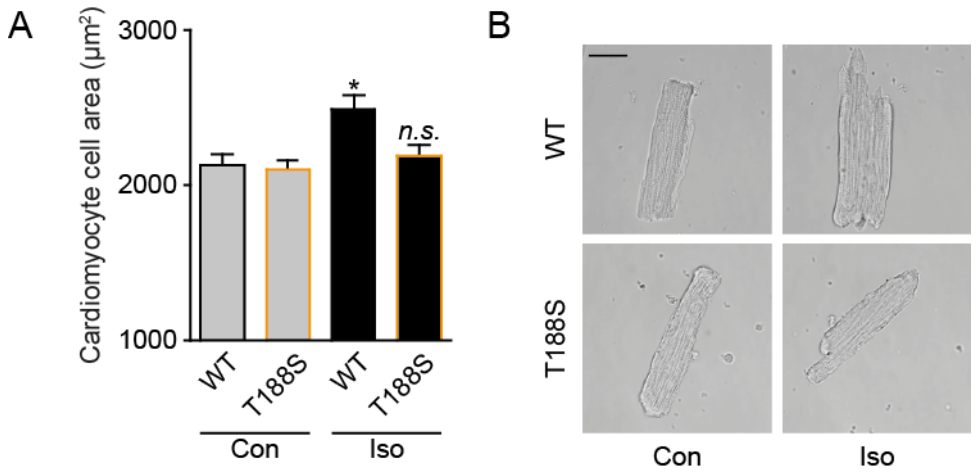



**Figure 33. Isoproterenol induced cardiomyocyte enlargement is attenuated in overexpressing Erk2$^{T188S}$ transgenic mice.** (**A**) Cell area measurements and (**B**) representative pictures of individual cardiomyocytes from wild type mice and overexpressing the phosphorylation deficient mutant Erk2$^{T188S}$ transgenic mice. CMs were treated without or with isoproterenol (5µM) for twelve hours. Scale bar represents 25µm. *n*=59-60 cells per animals, 4-5 animals per groups; *, *P*<0.05; *n.s. versus* unstimulated controls.

Cardiac hypertrophy is a risk factor for heart failure. Development of pathological hypertrophy is often accompanied with an up-regulation of gene expression and an increase in interstitial fibrosis. Cardiac fibrosis is an accumulation of extracellular matrix material in the cardiac interstitial space. This deposit is mediated by cardiac fibroblasts or abnormal regulation of collagen type 1 and 3 synthesis (Bernardo et al, 2010; Heineke & Molkentin, 2006; Muslin, 2008). Although echocardiographic analyses of the three genotypes, which are wild type mice and overexpressing wild type Erk2 and Erk2$^{T188S}$ transgenic mice, revealed no specific sign of heart failure, histological section stained with Sirius-red showed an increase in interstitial fibrosis in wild-type mice and overexpressing wild-type Erk2 (T188T) mice after isoproterenol treatment (Figure 34).
The histological sections were analyzed before and after osmotic mini-pump implantation. Since fibrosis is an accumulation of collagen, a common method to determine the increase in interstitial fibrosis is to stain the collagen with Sirius-red. Stained collagen can be then detected under a microscope and thus fibrosis is quantified. As shown in figure 34, transgenic mice overexpressing wild-type Erk2 (T188T), as well as wild-type mice, present increased interstitial fibrosis, while Erk2$^{T188S}$ transgenic mice (T188S) develop significantly less fibrosis.

To further confirm that in Erk2$^{T188S}$ transgenic mice the development of fibrosis is attenuated in response to isoproterenol treatment, mRNA expression levels of collagenase-3a, a marker of fibrosis (Dorn et al, 2003), were quantified. β-adrenergic receptor stimulation induced a significant increase in mRNA levels of collagenase-3a in wild-type mice and wild-type Erk2 overexpressing mice (T188T). In contrast to these results, mRNA levels of collagenase-3a in Erk2$^{T188S}$ overexpressing mice were notably attenuated (Figure 36) and correlated well with the quantification of fibrosis in cross-section of ventricular myocardium (Figure 34).

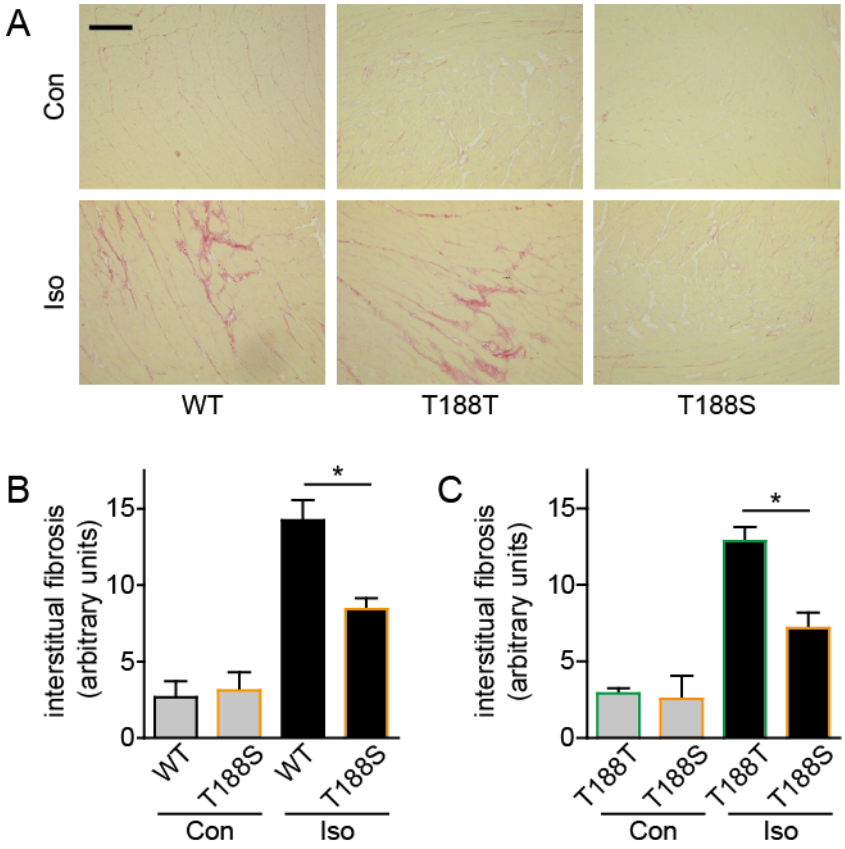



**Figure 34. Erk2$^{Thr188}$-phosphorylation elevates isoproterenol-induced fibrosis.** Histological analysis of ventricular myocardium of wild-type mice or transgenic mice overexpressing either wild-type Erk2 (T188T) or the phosphorylation deficient mutant Erk2$^{T188S}$ (T188S) before and after isoproterenol treatment (Iso; 30mg/kg/day). (**A**) Representative histological sections stained with Sirius-red and (**B**,**C**) quantification of interstitial fibrosis. Scale bar represents 100µm. (**B**) *n*=5-7 animals per group; (**C**) *n*=4-8 animals per group; *, *P*<0.01.

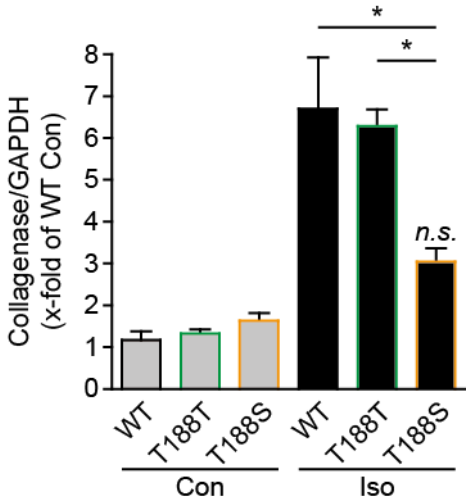



**Figure 35. Overexpression of Erk2$^{T188S}$ attenuates collagenase-3a expression after isoproterenol treatment.** mRNA levels of collagenase-3a in left ventricles of wild-type and transgenic mice overexpressing either wild-type Erk2 (T188T) or the phosphorylation deficient mutant Erk2$^{T188S}$ (T188S) before and after isoproterenol treatment (Iso; 30mg/kg/day) were determined by RT-PCR. *n*=4-10 animals per group; *, *P*<0.01.

Pathological hypertrophy and heart failure is also associated with an increase in fetal gene expression (Bernardo et al, 2010; Dorn et al, 2003; Lorenz et al, 2009a; Woodcock & Matkovich, 2005). Among these genes, some are encoding for atrial natriuretic peptides (ANP/ANF). Upon hypertrophic stimuli ANP levels tend to increase. Therefore quantification of these biomarkers is an interesting tool to determine whether a heart undergoes pathological hypertrophy.
Wild-type mice and wild-type Erk2 overexpressing mice (T188T) showed a significant increase in mRNA levels of ANP, whereas in transgenic Erk2$^{T188S}$ mice (T188S) ANP expression levels did not increase in response to isoproterenol treatment (Figure 36).

These results obtained *in vivo* confirm the important role of Erk2$^{Thr188}$-phosphorylation in β-adrenergic receptor induced cardiac hypertrophy.

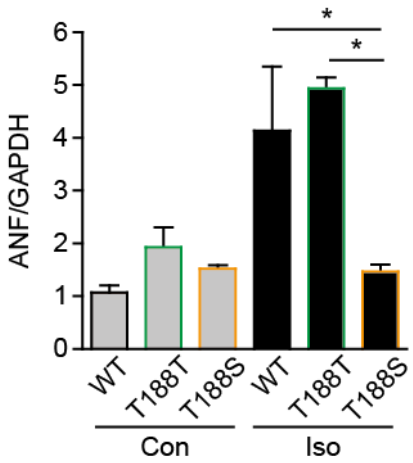



**Figure 36. The increase in ANF expression levels after isoproterenol treatment is attenuated by overexpression of Erk2$^{T188S}$.** mRNA levels of ANF in hearts of wild-type and transgenic mice overexpressing either wild-type Erk2 (T188T) or the phosphorylation deficient mutant Erk2$^{T188S}$ (T188S) before and after isoproterenol treatment (Iso; 30mg/kg/day) were determined by RT-PCR. *n*=4-10 animals per group; *, *P*<0.01.

# IV. Thr188-phosphorylated Erk is located in the nucleus and activates nuclear targets of Erk1/2

Upon activation, Erk1/2 have been demonstrated to phosphorylate several substrates localized either in the cytosol or in the nucleus of cardiomyocytes. Elk1 is a nuclear Erk1/2 target, which has been shown to be involved in cardiac hypertrophy (Bogoyevitch & Sugden, 1996; Frey & Olson, 2003; Heineke & Molkentin, 2006; Ramos, 2008). It has already been shown that $Erk2^{Thr188}$-phosphorylation triggers Elk1 phosphorylation upon $G_q$-protein coupled receptor activation (Lorenz et al, 2009a).

Since $Erk2^{Thr188}$-phosphorylation seems to play an important role in β-adrenergically mediated cardiac hypertrophy, the impact of $Erk2^{Thr188}$-phosphorylation on Elk1 phosphorylation was investigated by Western blot analysis. Left ventricles of transgenic mice with cardiac overexpression of either wild type Erk2 (T188T) or $Erk2^{T188S}$ (T188S) were collected without or with isoproterenol treatment (30mg/kg/day). Lysates of these samples were analyzed by Western blot using specific antibodies directed against phospho-Elk1. Western blots revealed a significant increase of Elk1 phosphorylation in transgenic mice overexpressing wild-type Erk2 (T188T) after isoproterenol treatment. However this isoproterenol mediated increase in Elk1 phosphorylation was inhibited in $Erk2^{T188S}$ transgenic mice (T188S) (Figure 37).

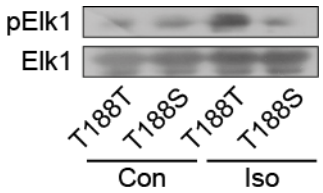



**Figure 37. Elk1 phosphorylation increases in transgenic mice overexpressing $Erk2^{Thr188}$-phosphorylation after isoproterenol treatment.** Representative immunoblots of heart lysates from transgenic mice overexpressing either wild-type Erk2 (T188T) or the phosphorylation deficient mutant $Erk2^{T188S}$ (T188S) before and after isoproterenol treatment (Iso; 30mg/kg/day). Specific antibodies directed against phospho-Elk1 and Elk1 were used.

Immunohistochemical analysis in heart sections of T188T and T188S transgenic mice confirmed the results obtained previously by Western blot analysis. Elk1 phosphorylation was detected using phospho-specific Elk1 antibodies and diaminobenzidine. Brown nuclei, therefore indicated activated Elk1. While brown staining was barely detectable in T188S transgenic mice before and after isoproterenol treatment (Figure 38, lower pictures), sections of transgenic mice overexpressing wild-type Erk2 (T188T) showed a significant Elk1 phosphorylation upon isoproterenol treatment (Figure 38, upper pictures).

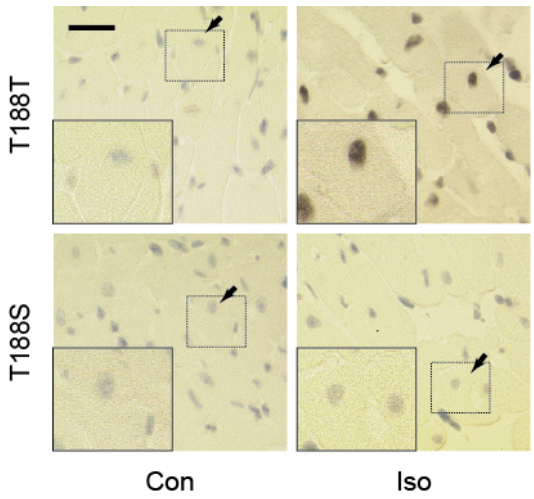



**Figure 38. Thr188-phosphorylated Erk triggers Elk1 phosphorylation.** Immunohistochemical analysis of Elk1 phosphorylation in heart sections of transgenic mice overexpressing either wild-type Erk2 (T188T) or the phosphorylation deficient mutant $Erk2^{T188S}$ (T188S) before and after isoproterenol treatment (Iso; 30mg/kg/day). Elk1 phosphorylation was detected using phospho-Elk1 antibodies and subsequent detection with diaminobenzidine. Scale bar represents 50µm.

Both immunoblot and immunohistochemistry analyses revealed that $Erk2^{Thr188}$-phosphorylation is important for Elk1 activation.

To activate Elk1, which is located in the cell nucleus, Erk1/2 needs to translocate to the nucleus (Ramos, 2008). We therefore examined whether T188S overexpression can retain Erk1/2 in the cytosol and thereby prevent translocation of Erk1/2 to the nucleus and thus the activation of its nuclear targets.

Erk2 localization was recorded by confocal microscopy before and after isoproterenol stimulation, using the yellow fluorescent protein (YFP). Thus, two constructs encoding for YFP-tagged wild type Erk2 (T188T) and YFP-tagged phosphorylation deficient mutant Erk2$^{T188A}$ (T188A) were generated.
For *in vitro* experiments both phosphorylation deficient mutants Erk2$^{T188A}$ and Erk2$^{T188S}$ were used (Figures 21, 22, 24 and 25). Since no significant differences were observed between them, Erk2 localization assays were performed with only YFP-tagged Erk2$^{T188A}$ mutant. The results were then extrapolated to Erk2$^{T188S}$.

Monkey kidney cells (COS-7) were transfected with either YFP-T188T or YFP-T188A and $\beta_2$-adrenergic receptors. After a 10-minute treatment with isoproterenol (5µM), COS-7 cells were fixed in paraformaldehyde solution and nuclei were stained with DAPI. Analyses of confocal microscopic pictures and quantification of nuclear-to-cytosolic distribution of YFP-Erk2 demonstrated that loss of Thr188-phosphorylation maintained Erk2 in the cytosol thereby preventing its translocation to the nucleus. Indeed, while YFP-T188A remained mainly in the cytosol, YFP-T188T translocated to the nucleus after isoproterenol treatment (Figures 39 and 40).

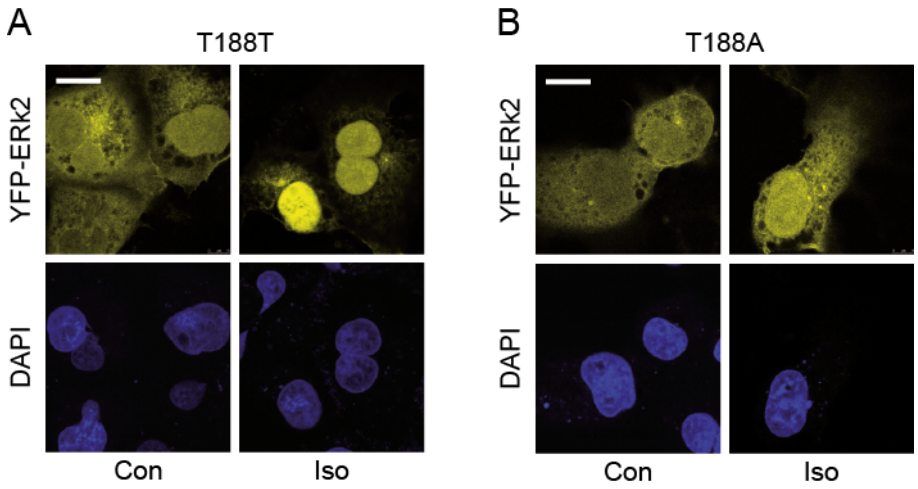



**Figure 39. Loss of Erk2$^{Thr188}$-phosphorylation prevents translocation of Erk2 to the nucleus.** Representative confocal pictures of nuclear-to-cytosolic distribution of YFP-tagged Erk2$^{T188T}$ (T188T) and YFP-tagged Erk2$^{T188A}$ (T188A). COS7 overexpressing either (**A**) YFP-T188T or (**B**) YFP-T188A and $\beta_2$-adrenergic receptors were treated without or with isoproterenol (Iso; 5µM) for 10 minutes. Nuclei were stained with DAPI (blue). Scale bar represents 20µm.

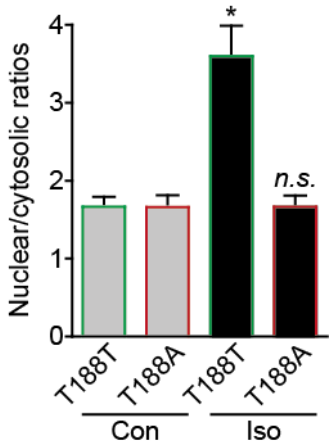



**Figure 40. Loss of Erk2$^{Thr188}$-phosphorylation retains Erk in the cytosol.** Ratios of nuclear-to-cytosolic distribution of YFP-tagged Erk2$^{T188T}$ (T188T) and YFP-tagged Erk2$^{T188A}$ (T188A). COS7 overexpressing either YFP-T188T or YFP-T188A and $\beta_2$-adrenergic receptors were treated without or with isoproterenol (Iso; 5µM) for 10 minutes. *n*=26-46 cells per group; *, *P*<0.001 *versus* all other conditions; *n.s. versus* unstimulated controls.

Nuclear-to-cytosolic distribution was also analyzed for YFP-Erk2$^{T188T}$ and YFP-Erk2$^{T188A}$ expressed in neonatal rat cardiomyocytes (NRCMs). Isolated NRCMs were transformed with adenoviruses encoding either for YFP-Erk2$^{T188T}$ (T188T) or YFP-Erk2$^{T188A}$ (T188A) and were then treated with isoproterenol (5µM) for 24 hours. Confocal microscopy confirmed that Thr188-phosphorylation is required for Erk translocation to the nucleus. Cells expressing YFP-T188T showed a clear localization of Erk in the nucleus after isoproterenol stimulation whereas YFP-T188A was only localized in the cytosol even upon isoproterenol stimulation (Figure 41). The results correlated with the experiments performed in COS7 cells.

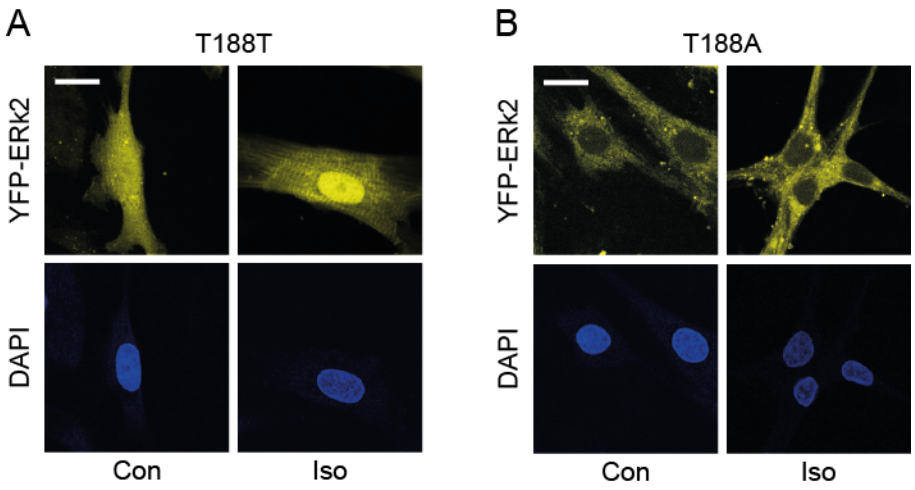



**Figure 41. Thr188-phosphorylated Erk2 translocates to the nucleus after β-adrenergic receptor activation.** Representative confocal pictures of the localization of YFP-tagged Erk2$^{T188T}$ (T188T) and YFP-tagged Erk2$^{T188A}$ (T188A) before and after isoproterenol treatment in NRCMs. NRCMs overexpressing either (**A**) YFP-T188T or (**B**) YFP-T188A were treated without or with isoproterenol (Iso; 5μM) for 24 hours. Nuclei were stained with DAPI (blue). Scale bar represents 20μm.

Taken together, these results demonstrate that activation of β-adrenergic receptors induces Erk2$^{Thr188}$-phosphorylation and that Erk$^{Thr188}$-phosphorylation leads to translocation of Erk to the nucleus, which in turn facilitates the activation of nuclear targets of Erk that can then induce cardiac hypertrophy.

# V. Activation of adenylyl cyclase is necessary but not sufficient for Thr188-phosphorylation of Erk to occur upon β-adrenergic receptor stimulation

$Erk2^{Thr188}$-phosphorylation plays a key role in mediating cardiomyocyte and cardiac hypertrophy upon β-adrenergic receptor activation (Figures 11-27). However the mechanism involved in this hypertrophic response is not clearly understood yet. β-adrenergic receptors signal mainly through the stimulatory G protein, $G\alpha_s$ (Bernardo et al, 2010; Frey & Olson, 2003; Lohse et al, 2003; Zheng et al, 2010) However, even if isoproterenol is a specific agonist of β-adrenergic receptors, crosstalk between different receptors signaling via other G protein such as $G\alpha_q$ or $G\alpha_i$ may occur and may thus also be responsible for the induction of $Erk2^{Thr188}$-phosphorylation of Erk1/2 and the effects of $Erk2^{Thr188}$-phosphorylation *in vitro* and *in vivo* (Figures 20-36).

To exclude any participation of $G\alpha_q$-coupled receptors expressed in NRCMs the specific $G\alpha_q$ inhibitor RGS2 (Hao et al, 2006; Heximer et al, 1997) was used. Indeed cardiomyocytes express endogenously several $G_q$-coupled receptors, such as $\alpha_1$-adrenergic or angiotensin II receptors, which could interfere with the effects of β-adrenergic receptors on Thr188-phosphorylation. NRCMs were transiently transduced with adenoviruses encoding for YFP-tagged RGS2 and compared to mock-transfected NRCMs. An involvement of $G\alpha_q$ in isoproterenol mediated Erk1/2 activity and hypertrophy was excluded by Western blot analysis (Figure 42) and [$^3$H]-isoleucine incorporation assays (Figure 43) respectively.
To control RGS2 efficiency on $G\alpha_q$ inhibition, NRCMs overexpressing RGS2 and mock-transfected cells were exposed to phenylephrine (2μM), a specific agonist of $\alpha_1$-adrenergic receptors (Figures 42B-43B). Indeed phenylephrine triggers Erk1/2 activation and cardiomyocyte hypertrophy through $\alpha_1$-ARs (Barki-Harrington et al, 2004; Kehat et al, 2011; Lorenz et al, 2009a).

Erk1/2 activity was analyzed by Western blot analysis using specific antibodies directed against the TEY motif. Mock transfected NRCMs showed a significant increase in Erk1/2 activity after both phenylephrine and isoproterenol treatment in comparison to untreated cells (Figure 42). Interestingly, phosphorylation at the TEY motif increased similarly in cardiomyocytes overexpressing RGS2 after

isoproterenol treatment (Figure 42A) but was notably inhibited after phenylephrine treatment (Figure 42B). These results excluded the role of $G\alpha_q$-protein in isoproterenol mediated Erk1/2 activation.

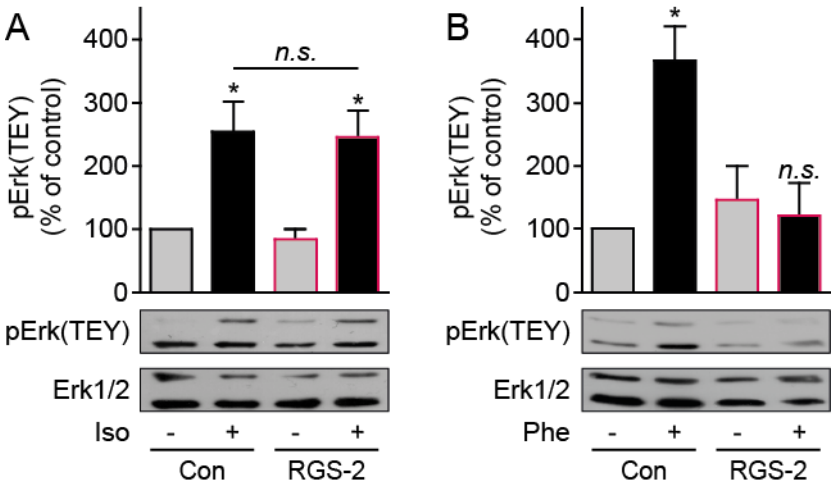



**Figure 42. Gq-protein is not involved in isoproterenol mediated Erk1/2 activation.** Representative immunoblots and their quantification using specific antibodies directed against the TEY motif [pErk(TEY)]. NRCMs overexpressing RGS2 were treated with (**A**) isoproterenol (Iso; 5µM) or (**B**) phenylephrine (phe; 2µM) for 10 minutes. Mock-transfected cells were used as additional controls. *n*=6-7 experiments; *, *P*<0.05 *versus* (**A**) unstimulated controls or (**B**) all other conditions; (**B**) *n.s. versus* unstimulated controls.

To determine whether $G_q$-protein activation interferes with the hypertrophic response of isoproterenol, NRCMs overexpressing RGS2 and mock-transfected cells were treated with isoproterenol (5µM) or phenylephrine (2µM) in presence of [$^3$H]-isoleucine (1µCi) for 30 hours.

A similar increase in cardiomyocyte hypertrophy was observed in mock-transfected cells and cells overexpressing RGS2 after isoproterenol treatment (Figure 43A), whereas RGS2 significantly inhibited the hypertrophic response to phenylephrine (Figure 43B).

Taken together, $G\alpha_q$ is neither involved in β-adrenergic induced Erk1/2 activation nor cardiomyocyte hypertrophy.

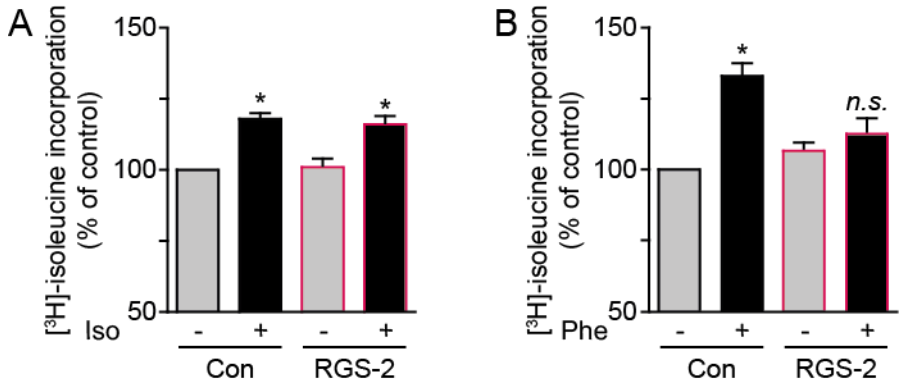



**Figure 43. While RGS2 inhibits phenylephrine induced hypertrophy, it has no effect on β-adrenergic receptor mediated hypertrophy.** [$^3$H]-isoleucine incorporation in NRCMs. Mock transfected or RGS2 overexpressing cells were treated with (**A**) isoproterenol (Iso; 5μM) or (**B**) phenylephrine (Phe; 2μM) in presence of ascorbic acid (100μM) and [$^3$H]-isoleucine (1μCi) for 30 hours. *n*=9-14 experiments; *, *P*<0.01 *versus* (**A**) unstimulated controls or (**B**) all other conditions; (**B**) *n.s. versus* unstimulated controls.

To analyze whether $G\alpha_i$ is involved in the induction of Erk2$^{Thr188}$-phosphorylation in response to β-AR activation, $G\alpha_i$ was inhibited by pretreatment of NRCMs with *pertussis toxin* (0.1μg/mL) (Hilal-Dandan et al, 2004; Morisco et al, 2001; Zheng et al, 2010) for 20 hours before isoproterenol treatment. Cell stimulated with carbachol (10μM) served as control, since carbachol leads to activation of Erk1/2 via $G\alpha_i$ coupled M2 muscarinic receptors in NRCMs (Lorenz et al, 2009a; Salazar et al, 2007; Wylie et al, 1999). Erk1/2 activity was detected by Western blot analyses using specific antibodies directed against the TEY motif. Pretreatment with *pertussis toxin* (PTX) did not attenuate isoproterenol mediated Erk1/2 activity (Figure 44A), whereas PTX pretreatment efficiently attenuated Erk1/2 activity upon carbachol stimulation (Figure 44B).
$G\alpha_i$-proteins therefore do not seem to be involved in β-adrenergically mediated Erk1/2 activity.

To confirm the importance of $G\alpha_s$ activation in Erk2$^{Thr188}$ mediated hypertrophy, the effects of adenylyl cyclase inhibition on Erk1/2 activity and cardiomyocyte hypertrophy were analyzed. Indeed adenylyl cyclase is the direct downstream target of $G\alpha_s$-proteins (Lohse et al, 2003; Rockman et al, 2002). Isolated NRMCs were therefore pretreated with KH7 (30μM), a specific inhibitor of adenylyl

cyclase (Kumar et al, 2009; Salinthone et al, 2011), 30 minutes prior to isoproterenol treatment (5µM).

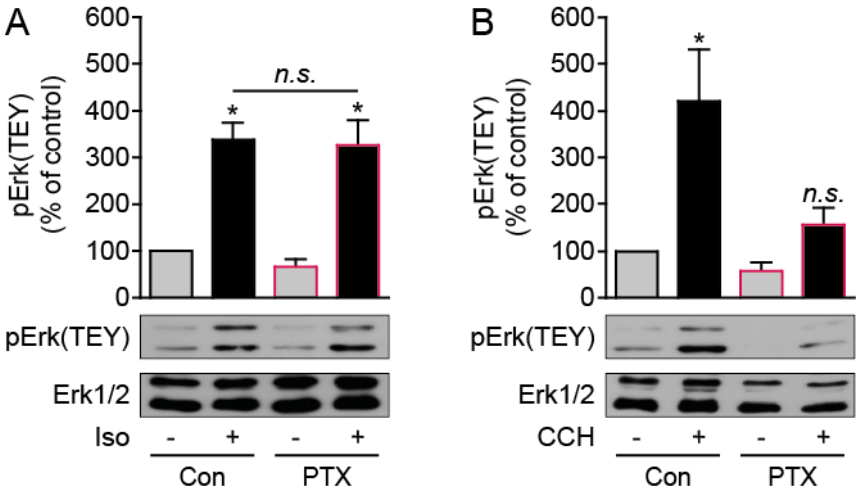



**Figure 44. $G_i$-protein activation is not involved in isoproterenol mediated Erk1/2 phosphorylation.** Representative immunoblots and their quantification using specific antibodies directed against the TEY motif [pErk(TEY)]. NRCMs were pretreated with *pertussis toxin* (PTX; 0.1µg/mL, 20h) and stimulated with (**A**) isoproterenol (Iso; 5µM) or (**B**) carbachol (CCH; 10µM) for 10 minutes. *n*=4-7 experiments; *, *P*<0.01 *versus* (**A**) unstimulated controls or (**B**) all other conditions; (**B**) *n.s. versus* unstimulated controls.

Erk1/2 phosphorylation at the TEY motif was detected by Western blot analyses (Figure 45) and cardiomyocyte hypertrophy was analyzed by [$^3$H]-isoleucine incorporation assays (Figure 46). KH7 pretreatment attenuated isoproterenol induced Erk1/2 activity (Figure 45) and hypertrophy (Figure 46).

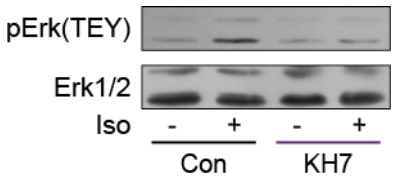



**Figure 45. Adenylyl cyclase activation is required for isoproterenol induced Erk1/2 activation.** Representative immunoblots using specific antibodies directed against the TEY motif [pErk(TEY)]. NRCMs were pretreated with KH7 (30µM; 30 minutes) and stimulated without or with isoproterenol (Iso; 5µM; 10 minutes).

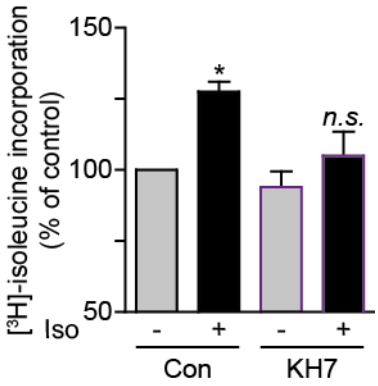



**Figure 46. Inhibition of adenylyl cyclase attenuates isoproterenol-induced cardiomyocyte hypertrophy.** [$^3$H]-isoleucine incorporation assay in NRCMs. Cells were pretreated with KH7 (30µM; 30 minutes) and treated without or with isoproterenol (Iso; 5µM) in presence of ascorbic acid (100µM) and [$^3$H]-isoleucine (1µCi) for 30 hours. *n*=5-7 experiments; *, *P*<0.05 *versus* all other conditions; *n.s. versus* unstimulated controls.

Even though adenylyl cyclase, and thus $G\alpha_s$ activation, is necessary for β-adrenergic receptor induced cardiomyocyte hypertrophy, adenylyl cyclase activation alone does not seem to be able to trigger cardiac hypertrophy (Frey & Olson, 2003). To verify this hypothesis adenylyl cyclase was directly activated with forskolin in NRCMs and cells were then monitored for cardiomyocyte hypertrophy as well as Erk1/2 activity.

NRCMs were treated either with isoproterenol (5µM) or forskolin (30µM) in presence of ascorbic acid (100µM) and [$^3$H]-isoleucine (1µCi/ml). Quantification of incorporated isoleucine confirmed that direct activation of adenylyl cyclase by forskolin does not trigger cardiomyocyte hypertrophy in contrast to isoproterenol (Figure 38). However, Erk1/2 was activated by both stimuli, isoproterenol as well as forskolin as monitored by phosphorylation of Erk1/2 at the TEY motif (Figure 48).

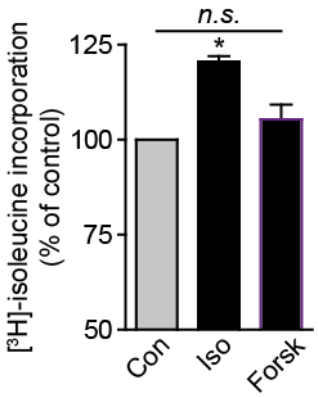



**Figure 47. Direct activation of adenylyl cyclase by forskolin does not induce cardiomyocyte hypertrophy.** [$^3$H]-isoleucine incorporation in NRCMs. Cells were treated with isoproterenol (Iso; 5µM) or forskolin (Forsk; 30µM) in presence of ascorbic acid (100µM) and [$^3$H]-isoleucine (1µCi/ml) for 30 hours. *n*=10 experiments; *, *P*<0.001 *versus* all other conditions.

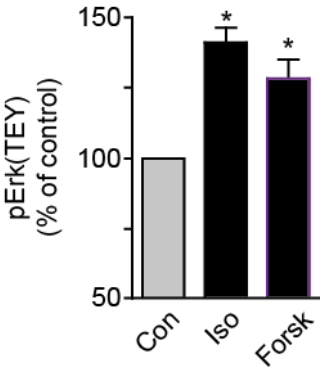



**Figure 48. Direct activation of adenylyl cyclase triggers Erk1/2 phosphorylation.** Quantification of immunoblot analysis using specific antibodies directed against the TEY motif [pErk(TEY)]. NRCMs were treated with isoproterenol (Iso; 5µM) or with forskolin (Forsk; 30µM) in presence of ascorbic acid for 24 hours. *n*=3-6 experiments; *, *P*<0.01 *versus* unstimulated controls.

Since Erk2$^{Thr188}$-phosphorylation is involved in β-adrenergic receptor induced hypertrophy, it was then analyzed whether direct activation of adenylyl cyclase could mediate Erk2$^{Thr188}$-phosphorylation. However, Western blot analyses of neonatal rat cardiomyocytes treated with forskolin revealed that direct activation of adenylyl cyclase was not sufficient to trigger Erk2$^{Thr188}$-phosphorylation (Figure 49).

In contrast to isoproterenol, forskolin was also not able to translocate YFP-Erk2 into the nucleus since forskolin treatment of cells overexpressing YFP-Erk2 wild-type retained Erk2 in the cytosol (Figure 50).

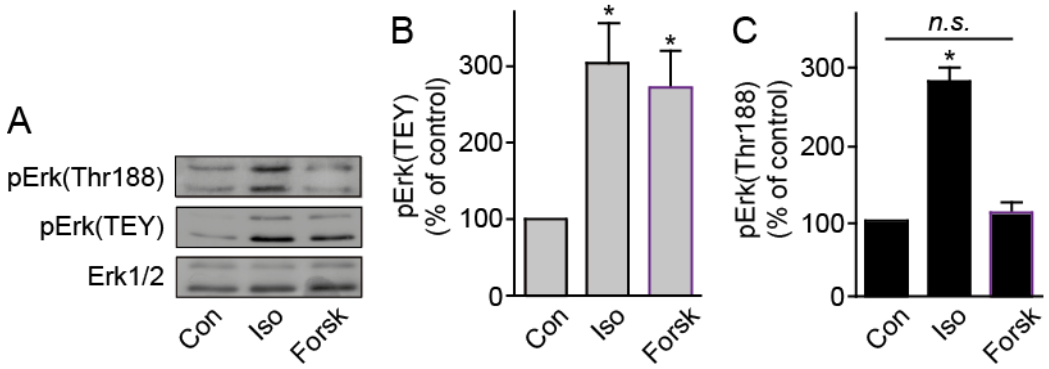



**Figure 49. Direct activation of adenylyl cyclase is not sufficient to induce Erk2$^{Thr188}$-phosphorylation.** (**A**) Immunoblot analyses and (**B**,**C**) their quantification using specific antibodies directed against (**B**) the TEY motif [pErk(TEY)] or (**C**) Thr188-phosphorylation [pErk(Thr188)]. NRCMs were treated with isoproterenol (Iso; 5μM) or with forskolin (Forsk; 30μM) for 10 minutes as indicated. *n*=4-6 experiments; *, *P*<0.05 *versus* (**B**) unstimulated controls or (**C**) all other conditions.

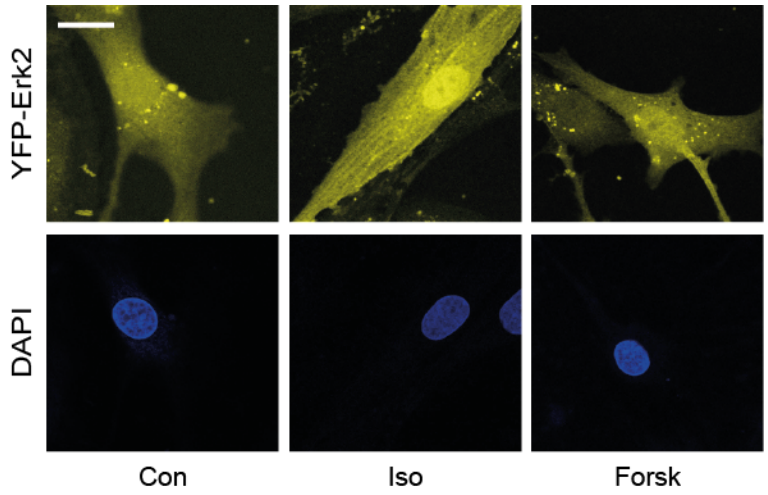



**Figure 50. Direct activation of adenylyl cyclase retains Erk2 in the cytosol.** Representative confocal pictures of nuclear-to-cytosolic distribution of YFP-tagged Erk2$^{T188T}$ (T188T). NRCMs overexpressing YFP-T188T were treated with isoproterenol (Iso; 5µM) or forskolin (Forsk; 30µM) when indicated for 24 hours. Nuclei were stained with DAPI (1:150). Scale bar represents 20µm.

Taken together these results demonstrate that AC activation is necessary but not sufficient to induce Erk2$^{Thr188}$-phosphorylation. Another event may therefore be involved in addition to AC activation for Erk2$^{Thr188}$-phosphorylation to occur. In their work, Lorenz et al. have shown that previous to Thr188-phosphorylation activated Erk1/2 bind directly with the βγ-subunits (Lorenz et al, 2009a). This association is maybe possible upon β-adrenergic receptors.

## VI. β-adrenergic receptors induce Erk2$^{Thr188}$-phosphorylation via a Gβγ dependent pathway

The ability of Erk and Gβγ-subunits to indeed form a complex upon β-adrenergic receptor activation was determined by co-immunoprecipitations assays in human embryonic kidney cells (HEK293).

HEK293 cells were transfected with Flag-tagged wild type Erk2 (T188T) and β1-adrenergic receptors. After a ten-minute exposition to isoproterenol (1µM), cells were lysed and an immunoprecipitation was performed by rotation of the lysate in presence of Protein A-sepharose beads pre-coated with anti-Flag antibodies for two hours at 4°C. Sepharose beads were then washed with ice-cold PBS and immunoprecipitated proteins were analyzed by Western blot analysis. Interestingly, Gβγ subunits co-precipitated with Flag-tagged Erk2 after isoproterenol stimulation (Figure 51).

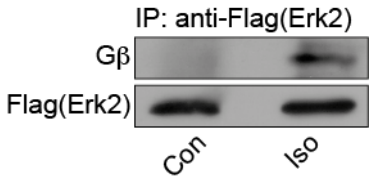



**Figure 51. $\beta_1$-adrenergic receptor activation mediates Gβγ/Erk2 complex formation.** Representative immunoblot of immunoprecipitated Flag-Erk2 and co-precipitated Gβγ. HEK293 cells overexpressing $\beta_1$-adrenergic receptors and Flag-Erk2 were treated without or with isoproterenol (Iso; 1µM) for 10 minutes. Flag-Erk2 were immunoprecipitated with Protein A-sepharose beads labeled with specific anti-Flag antibodies and co-precipitated Gβγ were detected using specific antibodies directed against Gβ.

Similarly, activation of $\beta_2$-adrenergic receptors over-expressed in HEK293 cells induced an association between Gβγ-subunits and HA-tagged wild-type Erk2. Immunoblot analyses of HEK293 cells treated with isoproterenol revealed an increase in Gβγ/Erk2 interaction, which was not detectable in non-treated cells (Figure 52).

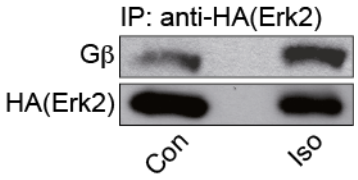



**Figure 52. $\beta_2$-adrenergic receptor activation mediates Gβγ/Erk2 complex formation.** Representative immunoblot of immunoprecipitated HA-tagged wild-type Erk2 and co-precipitated Gβγ. HEK293 cells overexpressing $\beta_2$-adrenergic receptors and HA-Erk2 were treated without or with isoproterenol (Iso; 1µM) for 10 minutes. HA-Erk2 were immunoprecipitated with Protein A-sepharose beads labeled with specific anti-HA antibodies and co-precipitated Gβγ were detected using specific antibodies directed against Gβ.

To support these results obtained in HEK293 cells, co-immunoprecipitation assays were conducted in NRCMs. Neonatal rat cardiomyocytes were transduced with adenoviruses encoding for Flag-tagged wild type Erk2. They were then treated 24 hours without or with isoproterenol (5µM) in presence of ascorbic acid (100µM). Phenylephrine (2µM), specific agonist of $G_q$-coupled $\alpha_1$-adrenergic receptors was used as positive control (Barki-Harrington et al, 2004; Kehat et al, 2011; Lorenz et al, 2009a). Following the same protocol as previously described for HEK293 cells, immunoprecipitated Flag-Erk2 and co-precipitated Gβγ subunits were detected by Western blots analysis.

While under basal conditions co-precipitated βγ-subunits were barely or not detectable, activation of β-adrenergic receptors triggered the association between Gβγ and overexpressed Flag-tagged Erk2 (Figure 53). The extent of Gβγ/Erk2 complex formation upon isoproterenol treatment was comparable to amount of co-immunoprecipitated Gβγ/Erk2 complex induced by phenylephrine (Figure 53).

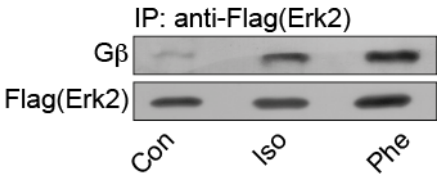



**Figure 53. Activation of endogenous β-adrenergic receptors triggers complex formation between Gβγ-subunits and overexpressed Flag-tagged Erk2.** Representative immunoblot of immunoprecipitated Flag-tagged wild-type Erk2 and co-precipitated Gβγ. NRCMs overexpressing Flag-Erk2 were treated with isoproterenol (Iso; 5μM) or phenylephrine (Phe; 2μM) in presence of ascorbic acid (100μM) for 24 hours. Flag-Erk2 were immunoprecipitated with Protein A-sepharose beads labeled with specific anti-Flag antibodies and co-precipitated Gβγ were detected using specific antibodies directed against Gβ.

The Erk1/2/Gβγ complex formation was also observed between endogenous Erk1/2 and Gβγ-subunits. Immunoblot analyses of co-immunoprecipitation assays performed in neonatal rat cardiomyocyte treated with isoproterenol revealed a complex formation between Erk1/2 and Gβγ-subunits (Figure 54). In line with overexpressed Flag-Erk2, the extent of interaction of endogenous Erk1/2 with Gβγ-subunits was similar in response to isoproterenol and phenylephrine treatment (Figure 54).

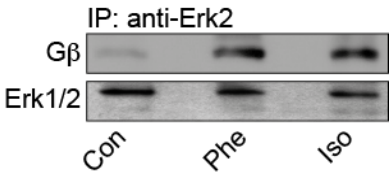



**Figure 54. Endogenous Erk1/2 interact with Gβγ-subunits upon β-adrenergic receptors stimulation.** Representative immunoblot of immunoprecipitated endogenous Erk1/2 and co-precipitated Gβγ. NRCMs were treated with isoproterenol (Iso; 5μM) or phenylephrine (Phe; 2μM) in presence of ascorbic acid (100μM) for 24 hours. Erk1/2 were immunoprecipitated with Protein A-sepharose beads labeled with p44/42 antibodies and co-precipitated Gβγ were detected using specific antibodies directed against Gβ.

Taken together these different co-immunoprecipitation assays reveal that activation of β-adrenergic receptors initiates a complex formation between Erk1/2 and the βγ-subunits. To support their importance in mediating Erk2$^{Thr188}$-phosphorylation, βγ-subunits were inhibited by overexpression of the Gβγ scavenger, the C-terminus of β-adrenergic receptor kinase (βark-ct) (Koch et al, 1994; Volkers et al, 2011). The effects of βγ-subunits inhibition on β-adrenergic receptor induced Erk2$^{Thr188}$-phosphorylation were observed in HEK293 cells.

HEK293 cells overexpressing $\beta_1$-adrenergic receptors and Flag-tagged wild type Erk2 were treated with isoproterenol in absence or presence of overexpressed βark-ct. Erk2$^{Thr188}$-phosphorylation was analyzed by Western blot using specific antibodies directed against pErk(Thr188). While phosphorylation at the TEY motif was increased similarly after isoproterenol stimulation in absence or presence of βark-ct, overexpression of βark-ct inhibited Erk$^{Thr188}$-phosphorylation completely (Figure 55).

Altogether these results confirm the importance of βγ-subunits in β-adrenergic receptor mediated Erk2$^{Thr188}$-phosphorylation.

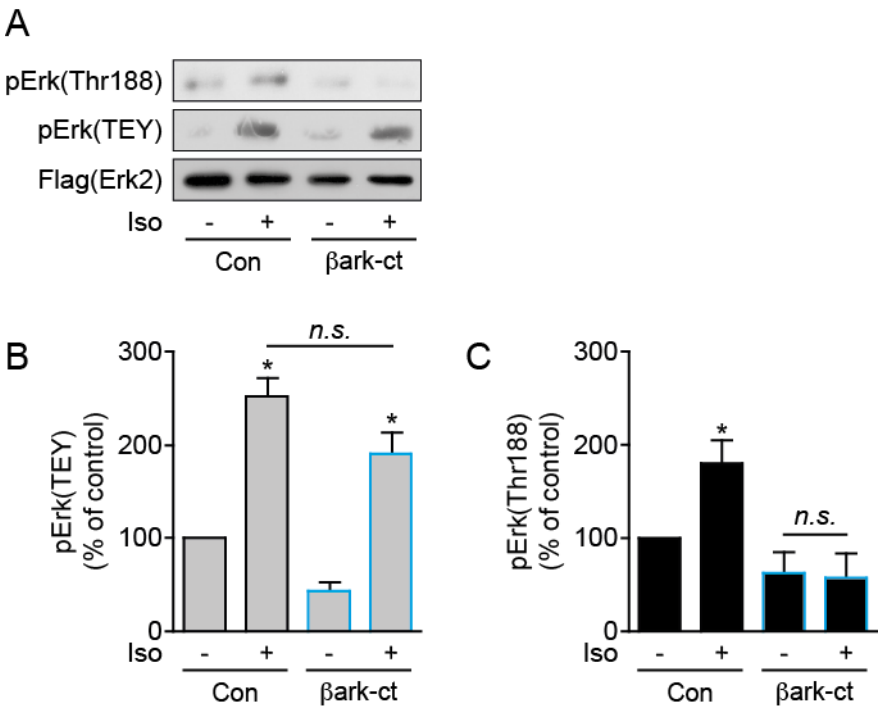



**Figure 55. Inhibition of Gβγ-subunits prevents Erk2$^{Thr188}$-phosphorylation.** (**A**) Representative Western blot of immunoblot analyses and (**B**,**C**) their quantification using specific antibodies directed against (**B**) the TEY motif [pErk(TEY)] or (**C**) Thr188-phosphorylation [pErk(Thr188)]. HEk293 cells overexpressing $\beta_1$-adrenergic receptors, Flag-tagged wild-type ERk2 and βarck-ct as indicated were treated with isoproterenol (Iso; 5μM) for 10 minutes. *n*=7-13 experiments; *, *P*<0.05 *versus* (**B**) unstimulated controls or (**C**) all other conditions.

# DISCUSSION

Taken together, the results suggest that β-adrenergically mediated cardiac hypertrophy derives from the convergence of two signaling pathways initiated by β-adrenergic receptors. On the one side, β-adrenergic receptors stimulate $G\alpha_s$ subunit leading to adenylyl cyclase activation and further canonical activation of the MAP kinase Erk cascade. On the other side, $G\beta\gamma$-subunits released from activated $G_s$ proteins bind to the activated Erk1/2 cascade. Coincidence of both events induces an autophosphorylation of Erk at threonine 188, responsible for the development of cardiac hypertrophy.

## I. β-adrenergic receptor signaling in cardiac hypertrophy

β-adrenergic receptors (β-ARs) play an important role in mediating cardiac hypertrophy and patients diagnosed with heart failure present an up-regulation of their sympathetic nervous system inversely correlated with their survival (Barki-Harrington et al, 2004; Frey & Olson, 2003; Lohse et al, 2003; Osadchii, 2007). Moreover β-adrenergic receptor antagonists, β-blockers, are widely used for the treatment of cardiovascular diseases (Baker et al, 2011). Nevertheless the signaling pathways involved in β-adrenergically induced cardiac hypertrophy are not yet fully understood and several questions remain.

Upon ligand binding β-ARs activate $G\alpha_s$ proteins, which in turn stimulate adenylyl cyclase (AC) leading to an increase in cAMP levels. The signal is then translated to protein kinase A (PKA), the main downstream target of cAMP and to Epac. Both proteins are then involved in the regulation of intracellular $Ca^{2+}$ (Métrich et al, 2010; Rockman et al, 2002). Even though the PKA-dependent pathway has been well documented and approved, it is not sufficient to explain the hypertrophic response of β-adrenergic receptors. While transgenic mice with a cardiac overexpression of either $\beta_1$-ARs or $G\alpha_s$ develop cardiac hypertrophy upon chronic catecholamine activation (Engelhardt et al, 1999; Iwase et al, 1997; Lohse et al, 2003), cardiac overexpression of adenylyl cyclase fails to promote heart growth (Roth et al, 2002) and 10-week old transgenic mice overexpressing PKA show

only a slight increase in ventricular wall thickness (Antos et al, 2001; Frey & Olson, 2003). In correlation with these results, direct activation of AC by forskolin does not – unlike β-AR activation – induce cardiomyocyte hypertrophy (Figure 48). These findings suggest that $G_s$ proteins may play a more important role than initially thought. An additional event involved in β-AR mediated cardiac hypertrophy may therefore occur at the level of $G\alpha_s$. Among their different downstream signaling effectors, β-ARs initiate the activation of the MAPK cascade Raf/Mek/Erk. Activation of Erk1/2 is mirrored by phosphorylation at threonine 183 and tyrosine 185, the so-called TEY motif. In both, isolated cardiomyocytes and murine heart tissues, direct activation of β-ARs induces canonical activation of Erk1/2 (Figures 9-14). In addition, direct activation of AC by forskolin triggers the phosphorylation of the TEY motif (Figure 48).

Several gain- and loss-of-function studies in transgenic mice have shown that the components of the Erk cascade promote cardiac hypertrophy *in vivo* (Kehat et al, 2011; Muslin, 2008; Rose et al, 2010). As an example, Bueno et al. generated transgenic mice with a cardiac overexpression of an activated Mek1 mutant. This study revealed that constitutive Erk1/2 activation in the heart is sufficient to induce cardiac hypertrophy (Bueno et al, 2000). However according to Purcell et al., complete inhibition of Erk is not sufficient to inhibit cardiac hypertrophy in response to various stimuli (Purcell et al, 2007). Erk1/2 are, therefore, significant components of cardiac hypertrophy signaling although their specific role is still an ongoing debate. Recently a new hypertrophic pathway has been described involving the classical Raf/Mek/Erk cascade together with an autophosphorylation of Erk2 at threonine 188. Convergence of both events is required to enhance cardiac hypertrophy in response to angiotensin as well as to pressure overload (Lorenz et al, 2009a). Since activated β-ARs trigger the canonical Erk cascade as well as $Erk2^{Thr188}$-phosphorylation in isolated cardiomyocytes and murine tissues (Figures 10-15), the hypothesis that a similar pathway may occur in the β-adrenergically triggered hypertrophic response was investigated.

## II. Effects of Erk2$^{Thr188}$-phosphorylation on cardiac functions

Inhibition of Erk1/2 activity, by the specific MEK inhibitor PD98059, prevents not only Erk2$^{Thr188}$-phosphorylation but also isoproterenol induced cardiomyocyte hypertrophy (Figure 17-18), suggesting ERK1/2 and ERK$^{Thr188}$-phosphorylation as possible players in β-AR mediated hypertrophy. Generation of Erk2 mutants that are deficient of the phosphorylation site at Thr188, Erk2$^{T188S}$ (T188S) and Erk2$^{T188A}$ (T188A), supported this hypothesis. While cardiomyocytes overexpressing wild-type Erk2 developed hypertrophy as mock transfected cells, the hypertrophic response was attenuated in NRCMs expressing T188S or T188A mutants upon β-adrenergic receptor stimulation (Figures 24-26). Cardiomyocyte hypertrophy is characterized by an increase in protein synthesis and enlargement of the cell surface area. Therefore, quantification of protein synthesis and cell area measurements are widely used methods to investigate hypertrophy *in vitro* (Dorn et al, 2003; Simpson & Savion, 1982; Woodcock & Matkovich, 2005). In cardiomyocytes overexpressing wild-type Erk2, the levels of protein synthesis (Figure 24) as well as the cell surface area (Figures 25 and 26) significantly increased after isoproterenol treatment. In comparison, inhibition of Erk$^{Thr188}$-phosphorylation mirrored by overexpression of Erk2$^{T188S}$ and Erk2$^{T188A}$ reduced the isoproterenol hypertrophic response (Figures 24-26). These different results obtained *in vitro* demonstrate that Erk2$^{Thr188}$-phosphorylation is involved in β-AR mediated cardiomyocyte hypertrophy and were further confirmed by *in vivo* studies.

The heart responds to variety of stimuli of the living organism, which is largely different to cell-culture and isolated cardiomyocyte experiments. Thus, an *in vivo* analysis of the signaling pathways in an organ, a living organism and mammals is indispensible to prove their significance under physiological conditions. (Hunter & Chien, 1999; Molkentin & Robbins, 2009; Woodcock & Matkovich, 2005). Therefore, animal models are used to support and validate cell culture experiments. For this purpose, transgenic mice overexpressing either wild-type Erk2 or the phosphorylation deficient mutant Erk2$^{T188S}$ were generated. Since no specific differences were recorded between the phosphorylation deficient mutants Erk2$^{T188S}$ and Erk2$^{T188A}$ *in vitro*, the *in vivo* experiments were only performed in Erk2$^{T188S}$ transgenic.

After sustained activation of β-ARs with isoproterenol, wild type mice and transgenic mice with cardiac overexpression of wild type Erk2 presented a significant increase in left ventricular wall thicknesses (Figures 27 and 28), cardiomyocyte size (Figure 32), fibrosis (Figures 34 and 35) and synthesis of atrial natriuretic peptides (Figure 38) characteristics of cardiac hypertrophy. In comparison, transgenic mice overexpressing phosphorylation deficient $Erk2^{T188S}$ were protected from cardiac hypertrophy (Figures 29-31).

Characteristics of cellular growth include also up-regulation contractile protein synthesis and sarcomere construction, leading to cytoskeletal reorganization. Sarcomeres can accumulate either in series to increase cell length and trigger eccentric hypertrophy or in parallel to increase individual cardiomyocyte width and induce concentric hypertrophy (Heineke & Molkentin, 2006; Hunter & Chien, 1999). Wild type mice showed a significant increase in septum and posterior wall thickness but no left ventricular dilatation after 14 days of isoproterenol treatment. Similar observations were obtained in transgenic mice with a cardiac overexpression of wild type Erk2. However $Erk2^{T188S}$ transgenic mice exhibited no hypertrophy (Figures 27-36). In addition, isoproterenol induced the enlargement of adult cardiomyocytes isolated from wild-type mice (Figure 33) Sarcomere reorganization in parallel was also observed in phalloidin-stained NRCMs overexpressing wild type Erk2 upon isoproterenol treatment (Figure 25). These findings suggest that $Erk^{Thr188}$-phosphorylation may induce concentric hypertrophy, which correlate well with previous studies. Kehat et al. have recently shown that Erk1/2 regulate the balance between eccentric and concentric cardiac hypertrophy. Upon hypertrophic stimuli activated Erk initiates an increase in individual cardiomyocyte width, while loss of Erk1/2 activity induces eccentric hypertrophy (Kehat et al, 2011).
Altogether *in vitro* and *in vivo* data demonstrate that $Erk^{Thr188}$-phosphorylation mediated by β-adrenergic receptors is actively involved in β-adrenergically induced cardiac hypertrophy.

Cardiac hypertrophy can contribute to the development of heart failure. However, after two weeks of isoproterenol treatment no sign of heart failure or deterioration of the heart function was observed in the mouse model used in this work (Figures 29-31). Therefore, it was impossible to determine the role of $Erk2^{Thr188}$-phopshorylation on chronic β-adrenergic receptor activation mediated heart failure. However, interference with $Erk^{Thr188}$-phosphorylation by

overexpression of the phosphorylation deficient mutant $Erk2^{T188S}$ reduced interstitial fibrosis and ANF expression levels. Even if the impact of $Erk2^{Thr188}$-phosphorylation on β-adrenergically induced heart failure remains unclear, its blockade could raise new therapeutic interests in the treatment of β-ARs mediated cardiac hypertrophy. These findings could be explained by the role of Erk, which is quite complex. Indeed Erk1/2 regulate several biological processes. Previous studies have shown that Erk play a antiapoptotic role in the heart (Kehat & Molkentin, 2010). These protective effects may counteract the hypertrophic detrimental effects.

## III. Convergence of two signaling cascades

β-adrenergic receptors induce Erk1/2 activation via their $G\alpha_s$-proteins (Zheng et al, 2010). However several elements indicate that this pathway alone is not sufficient to mediate $Erk2^{Thr188}$-phosphorylation. Even though direct activation of AC by forskolin induces Erk phosphorylation at the TEY motif, it fails to initiate $Erk2^{Thr188}$-phosphorylation and cardiomyocyte hypertrophy (Figures 47-49). In addition, inhibition of adenylyl cyclase using the inhibitor KH7 blocks Erk1/2 activity and isoproterenol mediated cardiomyocyte hypertrophy completely (Figures 45 and 46). These results confirm that downstream events of $G\alpha_s$ are mandatory for Erk1/2 activation but are not sufficient to induce $Erk2^{Thr188}$-phosphorylation and cardiac hypertrophy. Another pathway is therefore required simultaneously.

In parallel, co-immunoprecipitation assays show that both $\beta_1$-and $\beta_2$-adrenergic receptors initiate a direct binding between Erk1/2 and $G\beta\gamma$-subunits (Figures 51-54). Sequestration of βγ-subunits by overexpression of βark-ct (Volkers et al, 2011) impedes $Erk2^{Thr188}$-phosphorylation but has no effect on Erk1/2 activity (Figure 55). These findings are in line with previous reports and confirm the importance of βγ-subunits. Direct binding of activated Erk1/2 and $G\beta\gamma$-subunits is a prerequisite for $Erk2^{Thr188}$-phosphorylation to occur (Lorenz et al, 2009a).

These results support the idea that $Erk2^{Thr188}$-phosphorylation derives from the convergence of two distinct pathways ($G\alpha_s$ and $G\beta\gamma$) initiated by the same receptor. Upon ligand-binding β-adrenergic receptors activate $G_s$-proteins. $G\alpha_s$-subunits initiate activation of the Erk1/2 cascade via adenylyl cyclase and its classical downstream pathway, leading to phosphorylation at the TEY motif and

Erk dimerization. Simultaneously, Gβγ-subunits detach from the G proteins and bind to the entire MAPK cascade. The coincidence of both events leading to Gβγ/ERK binding triggers Erk2$^{Thr188}$-phosphorylation, which then leads to the activation of diverse transcription factors responsible for cardiac hypertrophy (Figure 55).

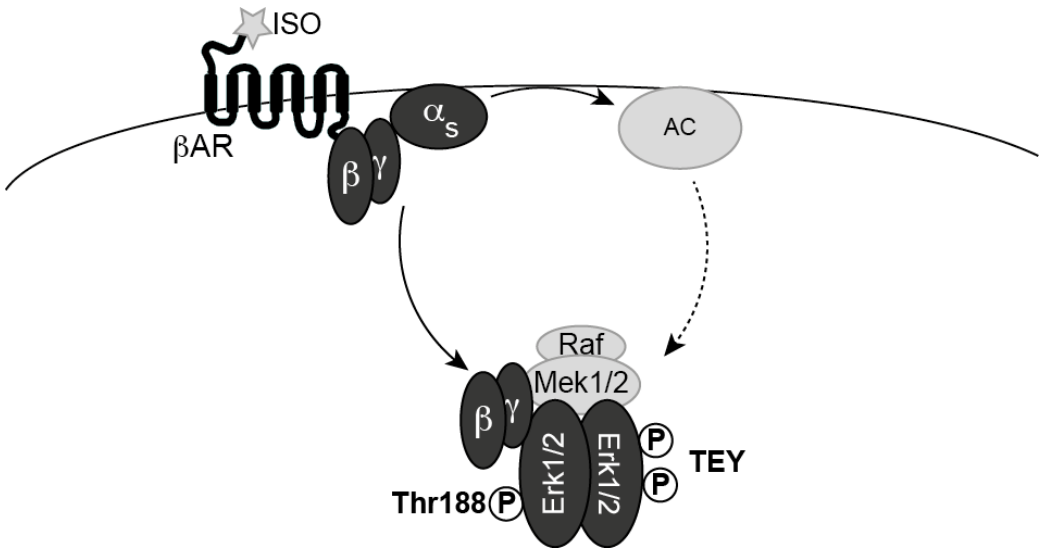



**Figure 56. Erk$^{Thr188}$-phosphorylation results from the convergence of two pathways.** Activation of β-adrenergic receptors triggers canonical activation of the Erk cascade through the classical $G\alpha_s$ pathway leading to phosphorylation at the TEY motif, while Gβγ-subunits released from activated $G_s$ bind to the MAP kinase cascade. Convergence of both events mediates Erk2$^{Thr188}$-phosphorylation, which participates strongly in cardiac hypertrophy.

## IV. Nuclear localization of Erk

Activation of the Erk cascade results from a wide spectrum of stimuli and can itself initiate diverse signaling pathways. However some characteristics regulate the specificity of the cascade and the specificity of the biological response. Depending on their subcellular localization, Erk1/2 activate different substrates. In resting cells, components of the Erk cascade are mainly localized in the cytosol while after activation 60-70% of the molecules translocate to the nucleus.

The remaining 30-40% of active Erk1/2 either stay in the cytosol or translocate in other organelles (Zehorai et al, 2010).

Although the mechanisms involved in nuclear translocation of Erk1/2 are not fully understood yet, it has been shown that Erk dimerizes upon Mek activation in a phosphorylation-dependent manner and translocates to the nucleus. Even if both Erk dimerization and Erk activation are necessary (Adachi et al, 1999; Caunt & McArdle, 2012; Cobb & Goldsmith, 2000; Khokhlatchev et al, 1998), those triggers seem not sufficient to induce Erk nuclear translocation: Direct activation of adenylyl cyclase with forskolin leads to Erk phosphorylation at the TEY motif (Figures 48 and 49), but Erk is retained within the cytosol (Figure 50). Since forskolin is not able to trigger $Erk2^{Thr188}$-phosphorylation (Figure 49), these findings suggest that nuclear accumulation of Erk requires Thr188-phosphorylation. Further localization analyses of Erk2 in COS7 cells (Figures 38 and 39) or in cardiomyocytes (Figure 40) confirmed this hypothesis. The phosphorylation deficient mutant $Erk2^{T188A}$ was unable to translocate into the nucleus in contrast to wild type Erk2 even after β-adrenergic receptors activation. $Erk^{Thr188}$-phosphorylation is therefore mandatory for Erk to enter the nucleus. However the mechanisms leading to nuclear accumulation of $Erk^{Thr188}$-phosphorylation are still unknown.

Elk1 is a nuclear target of Erk1/2, which participates strongly in mediating cardiac hypertrophy (Frey & Olson, 2003; Heineke & Molkentin, 2006; Ramos, 2008). The fact that activation of Elk1 is attenuated by overexpression of $Erk2^{T188S}$ (Figures 37 and 38) underlines the importance of $Erk^{Thr188}$-phosphorylation in the nuclear translocation of Erk and its involvement in mediating hypertrophic downstream pathways.

Like wild-type Erk1/2, the phosphorylation deficient mutants $Erk2^{T188S}$ and $Erk2^{T188A}$ can dimerize (Lorenz et al, 2009a). They can form either homodimers or heterodimers with wild-type Erk1 and Erk2. Cytosolic sequestration of Erk1/2 may therefore result from a dominant negative effect of the phosphorylation deficient mutants. This dominant negative effect has been recently shown (Ruppert et al, 2013). Indeed $Erk2^{T188A}$ mutants form heterodimers with endogenous Erk1/2 and retain them within the cytosol. Since Erk1/2 are sequestered within the cytosol and can no more accumulate within the nucleus, they do not activate their nuclear targets. However Erk1/2 are still able to

phosphorylate their cytosolic targets such as p90 ribosomal S6 kinase (p90RSK) (Ruppert et al, 2013). Interestingly nuclear targets of Erk1/2, such as Elk1, initiate cardiac hypertrophy while p90RSK promotes a protective antiapoptotic signaling.

It remains to elucidate the cellular localization of the βγ/Erk complex formation. Since Gβγ-subunits are commonly localized at the cell membrane close to the receptors, it would be interesting to know where Gβγ interact with Erk and how Thr188-phosphorylated Erk translocates to the nucleus.

## V. G protein specificity

A similar hypertrophic pathway has been previously described for $G_q$-coupled receptors. Upon angiotensin receptor activation, the Gβγ-subunits of activated $G_q$ proteins dissociate to form a complex with the canonically activated Erk cascade. This direct interaction leads to the autophosphorylation of Erk2 at threonine 188 responsible for hypertrophic response of angiotensin (Lorenz et al, 2009a; Lorenz et al, 2009b). However, this pathway is not specific to angiotensin receptors but can results from other $G_q$-coupled receptors such as the $\alpha_1$-adrenergic receptor. Since several $G_q$-coupled receptors induce $Erk2^{Thr188}$-phosphorylation, such as angiotensinII, muscarinic1 (Lorenz et al, 2009a) or $\alpha_1$-adrenergic receptors (Figures 11, 12 and 22), it would be interesting to know whether $Erk2^{Thr188}$-phosphorylation is specific for β-adrenergic receptors or whether others, if not all, $G_s$-coupled receptors also mediate this signaling pathway. In the heart $G_s$-coupled receptors also include adenosine, glucagon, prostanoid and histamine receptors (Salazar et al, 2007).

Direct binding between Gβγ-subunits and Erk is a necessary prerequisite for $Erk^{Thr188}$-phosphorylation to occur (Lorenz et al, 2009a). Preliminary assays in HEK293 cells overexpressing $G_s$-coupled histamine2 receptors and Flag-tagged Erk2 showed that the Gβγ subunits interact directly with Erk upon histamine activation (data not shown in this work). Interestingly the Gβγ/Erk complex forms upon stimulation of $G_q$-coupled histamine1 receptors but not upon $G_i$-coupled histamine3 receptor activation. These preliminary observations were in line with previous reports (Lorenz et al, 2009a). While activation of $G_q$-coupled angiotensin receptors triggers a direct interaction between Gβγ-subunits and Erk in isolated cardiomyocytes, stimulation of $G_i$-coupled $M_2$ muscarinic receptors does not (Lorenz et al, 2009a). Even if more experiments are required, these preliminary

results support therefore the idea of a similar pathway for $G_q$- and $G_s$-coupled receptors but not for $G_i$-coupled receptors.

Several studies have revealed that upon activation all G proteins dissociate and that all of the subunits can interact independently with their effectors (Clapham & Neer, 1997; Dupre et al, 2009; Lin & Smrcka, 2011). However this common dogma of subunit dissociation has been recently discussed. Using the fluorescence resonance energy transfer (FRET) method, Frank et al. revoked the idea that all G proteins dissociate. They showed that $G\alpha_{i1,2,3}$ and $G\alpha_z$ are not able to detach from their $G\beta\gamma$-subunits in comparison to $G\alpha_o$ (Frank et al, 2005). Since activation of $G\alpha_i$-coupled receptors fails to induce $Erk^{Thr188}$-phosphorylation (Lorenz et al, 2009a), it would be interesting to investigate the role of G protein dissociation in this signaling pathway. Indeed, it is still not clear whether $G\beta\gamma$-subunits need to detach from the $G\alpha$-subunit to form a complex with Erk1/2. Overexpression of $G\alpha_o$ together with $G\alpha_i$-coupled receptors, such as histamine3 would maybe initiate the $\beta\gamma$/Erk complex formation and thus, $Erk^{Thr188}$-phosphorylation, while overexpression of $G\alpha_i$ would prevent it. These experiments could also be performed in cardiomyocytes since adenosine1, muscarinic2 and $\alpha_2$-adrenergic receptors, which couple to $G\alpha_i$, are naturally expressed in the heart (Salazar et al, 2007). Such findings would bring new understanding of the pathway. $G\beta\gamma$-subunits are required for induction of $Erk^{Thr188}$-phosphorylation (Figure 56) and this phosphorylation participates actively in the development of cardiac hypertrophy (Figures 21-37) and (Lorenz et al, 2009a). Inhibition of G protein dissociation could possibly impede $Erk^{Thr188}$-phsophorylation and, thus, cardiac hypertrophy without disturbing classical adenylyl cyclase activation and its downstream signaling pathway. Indeed dissociation is not mandatory for good G protein function. Even if $G\alpha_s$ proteins do not dissociate, adenylyl cyclase activation is intact (Levitzki & Klein, 2002). Moreover the MAP kinase Erk cascade is involved in many biological processes, such as cell differentiation, proliferation, migration and more (Kehat & Molkentin, 2010; Rose et al, 2010; Seger & Krebs, 1995; Shaul & Seger, 2007). Blocking the whole cascade could thus inhibit important downstream beneficial events and trigger armful side effects. Inhibition of G protein dissociation could maybe raise new strategies for cardiac hypertrophy therapy.

# VI. Targeting strategies

The G protein dissociation is not the only possible event that brings similarities between $G_q$- and $G_s$-coupled receptors. Indeed, $G\alpha_q$ and $G\alpha_s$ trigger similar downstream events. For instance activation of both proteins increases $Ca^{2+}$ levels (Tilley, 2011). As described previously, stimulation of $G\alpha_s$-proteins by β-adrenergic receptors induces protein kinase A (PKA) activation. Via phosphorylation of L-type calcium channels and ryanodine receptors, PKA signaling triggers extracellular $Ca^{2+}$ influx and $Ca^{2+}$ release from the sarcoplasmic reticulum (Heximer et al, 1999) release, leading to $Ca^{2+}$/calmodulin-dependent protein kinase II (CaMKII) activation (Frey & Olson, 2003; Grimm & Brown, 2010; Tilley, 2011). Ligand binding on $G_q$-coupled receptors also initiates $Ca^{2+}$ SR release and CaMKII activation: Phospholipase C, the direct effector of $G_q$-proteins mediates the generation of inositol-1,4,5-trisphosphophate, involved in $Ca^{2+}$ SR release. Interestingly $G\alpha_i$-proteins do not participate in such events. Via adenylyl cyclase (AC) inhibition, $G\alpha_i$-proteins impede second messenger production and downstream signaling pathway of AC (Tilley, 2011). Since $Ca^{2+}$ and CaMKII are activated upon $G_q$- or $G_s$-protein signaling pathway only, they may play a role in $Erk2^{Thr188}$-phosphorylation. Elucidating the molecule or the cellular events where both pathways converge would bring new understanding of the mechanisms involved in mediating $Erk2^{Thr188}$-phosphorylation.

In addition to the wide spectrum of upstream signaling events mediating Erk1/2 activation, Erk1/2 itself participates strongly in different downstream mechanisms. Even if $Erk2^{Thr188}$-phosphorylation is involved in β-adrenergic receptor mediated cardiac hypertrophy (Figures 20-36), the exact mechanisms connecting $Erk2^{Thr188}$-phosphorylation to cardiac hypertrophy are not yet completely understood. $Erk^{Thr188}$-phosphorylation seems required for nuclear localization of Erk (Figures 39-41 and (Lorenz et al, 2009a) and for phosphorylation of nuclear Erk targets known to induce cardiac hypertrophy, such as Elk1, mitogen- and stress-activated protein kinase-1 (MSK1) and c-Myc (Figures 37 and 38; and (Lorenz et al, 2009a)). It would be then interesting to investigate whether $Erk^{Thr188}$-phosphorylation regulates other downstream signaling pathways involved in β-AR mediated cardiac hypertrophy. In the failing heart, the activity and the expression levels of CaMKII and Epac are up-regulated. Moreover, both proteins have been shown to mediate hypertrophy through β-AR activation

(Mangmool et al, 2010; Metrich et al, 2008). It is, therefore, conceivable that Erk$^{Thr188}$-phosphorylation may play a role in those hypertrophic pathways by modulating CaMKII and Epac activation.

Most of the current heart failure treatments target β-adrenergic receptors (β-blockers). Recent studies have shown that in addition to their antagonistic effects on the deleterious G protein signaling pathway, β-blockers also signal through an independent G protein pathway involving β-arrestin (Noor et al, 2011; Tilley, 2011). This new G protein independent β-arrestin dependent pathway also leads to Erk1/2 activation.
Interestingly, activation of Erk1/2 by β-arrestin2 leads to sequestration of Erk in the cytosol (Cervantes et al, 2010). Indeed β-arrestin interacts directly with Erk and thus impedes its translocation to the nucleus. Erk, retained in the cytosol, can no more phosphorylate its nuclear substrates. Following the same line, direct binding of overexpressed β-arrestin2 with Erk1/2 prevents the formation of the Gβγ/Erk1/2 complex and, thus, Erk2$^{Thr188}$-phosphorylation (Ruppert et al, 2013). These findings suggest that β-arrestin could be an interesting tool to inhibit the detrimental effects of Erk$^{Thr188}$-phosphorylation. It would be then interesting to investigate whether some β-blockers selectively activate the β-arrestin dependent pathway while blocking the G protein dependent pathway.

Several studies performed either in cardiomyocytes or in transgenic mice have shown that Erk1/2 play an important role in the regulation of cardiac hypertrophy. The introduction of Erk$^{Thr188}$-phosphorylation and its involvement in mediating cardiac hypertrophy follows the same direction. These findings suggest that inhibition of Erk1/2 can prevent cardiac growth. However, the relationship between the MAPK cascade and the hypertrophic response is not that straightforward. First of all, Erk1/2 control several cellular processes: For example, they play an important cardioprotective, anti-apoptotic role (Rose et al, 2010). Transgenic mice with a cardiac overexpression of constitutively active Mek1 are protected from injury and apoptosis in response to ischemia-reperfusion injury (Bueno et al, 2000). Inhibition of Erk1/2 could, thus, protect the heart from hypertrophy but it could also trigger severe side effects. Moreover the response of Erk from any biological stimulus depends on different parameters, such as the duration and strength of the signal, its interaction with different scaffold proteins, its subcellular localization and cross-talk possibilities with other

cascades (Kehat & Molkentin, 2010; Zehorai et al, 2010). Therefore instead of targeting the entire Raf/Mek/Erk cascade it would be better to modulate these parameters. For instance, $Erk^{Thr188}$-phosphorylation induces accumulation of Erk within the nucleus and, thus, Elk1 activation. Prevention of this translocation could maybe impede only with the hypertrophic effects of Erk1/2 and preserve their cardioprotection effects. Lorenz et al. have also shown that $Erk^{Thr188}$-phosphorylation is more sustained than phosphorylation at the TEY motif (Lorenz et al, 2009a). The duration of the signal is maybe an interesting point to investigate. Moreover the direct binding of Gβγ-subunits with the MAPK cascade is a prerequisite for $Erk^{Thr188}$-phosphorylation to occur. Disruption of the complex formation could also be an interesting perspective to specifically impede the hypertrophic effects of $Erk^{Thr188}$-phosphorylation.

# VII. Role of $Erk2^{Thr188}$-phosphorylation in other diseases

The Raf/Mek/Erk cascade is ubiquitous and regulates several cellular events. It is indeed involved in several biological and physiological events. Among these processes the MAP kinase cascade initiates cell proliferation, survival and differentiation. Therefore deregulations or mutations of the components involved in the Erk cascade are often linked with a wide range of diseases, such as several types of cancers (Lawrence et al, 2008; Mebratu & Tesfaigzi, 2009; Montagut & Settleman, 2009; Roberts & Der, 2007). Even if cancer therapy studies have mainly been focused on the PI3K signaling pathway and its role in cell survival, new evidences have raised that suggest the Erk cascade is a key component and a good target for cancer treatment (Balmanno & Cook, 2009). Indeed Ras mutations have been reported in pancreatic (90%), colon (50%), thyroid (50%), lung (30%) and melanoma (25%) cancers, while B-Raf is mutated in 60% of malignant melanomas and some other human cancers such as colon (20%) or ovary (30%). Both types of mutations are sufficient to promote a persistent activation of Erk1/2 and, thus, contribute to increased tumor cell proliferation (Mebratu & Tesfaigzi, 2009; Montagut & Settleman, 2009). Inhibition of the MAPK signaling pathway has become a promising therapeutic strategy in cancer treatment. However, even though diverse Ras, Raf or even Mek inhibitors have been described, tested and some of them are now available in the clinic, several problems remain such as toxicity and efficacy. Sorafenib, a Raf kinase

inhibitor was approved by the Food and Drug Administration and the European Medicines Agency for the treatment of advanced renal cell carcinoma and advanced hepatocellular carcinoma. Patients treated with sorafenib show an increase in approximatly 3 months of progression-free survival. Even though it is considered as a safe and well-tolerated drug, several side effetcs have been reported such as diarrhea and hand-food skin toxicity. Examples of Mek inhibitors are Cl-1040 and PD0325901. Phase II studies revealed that Cl-1040 was well tolerated but not clinically sufficient. In comparison, PD0325901 showed promising preclinical activity but was stopped in phase I because of unacceptable ocular toxicity (Montagut & Settleman, 2009; Roberts & Der, 2007). Overall targeting of the Raf/Mek/Erk cascade is a challenging task because of its various physiologocal functions.

Depending on the cell type and the stimuli involved, Erk activation induces different subcellular localization and different cellular responses. For instance in the nucleus, phosphorylated Erk activates either Elk1, which in turn enhances transcription of c-Fos, a growth relative protein. In some other cells, the death associated protein kinase (DAPK) sequesters Erk in the cytosol, preventing further nuclear translocation and, thus, proliferation. Moreover Erk phosphorylates DAPK, which mediates apoptosis (Mebratu & Tesfaigzi, 2009). These two examples show the correlation between Erk localization and the cellular response. It would, therefore, be interesting to investigate a targeting strategy that keeps Erk within the cytosol and thereby targets only specific responses. Cytosolic Erk sequestration would induce apoptosis of tumor cells and prevent proliferation and cell survival. Such a pathway could block detrimental effects of Erk but enhance the beneficial effects.

In addition, mutations, stimulation of receptor tyrosin kinases or GPCRs also enhances tumor cell proliferation and survival (Montagut & Settleman, 2009). Some of these receptors and their ligands are highly concentrated in cancerous tissues. For instance, lysophosphatidic acid (LPA) receptors, $G_q$-signaling, are expressed in ovarian, breast, colon and prostate cancer, whereas endothelin receptors, $G_q$-signaling, exists in colon and prostate cancers as well as melanoma (Hurst & Hooks, 2009). $G\alpha_i$ and $G\alpha_s$ have also been found in human endocrine and pituitary tumors respectively.
It would therefore be interesting to investigate for $Erk^{Thr188}$-phosphorylation on its role in tumor progression and to determine whether cancer cell proliferation can

be enhanced by Erk$^{Thr188}$-phosphorylation. Following the same argument, the role of Thr188-phosphorylated Erk in cancer cell survival or migration may an important field of investigation. Upon activation, Thr188-phosphorylated Erk translocates to the nucleus and phosphorylates Elk1 involved in cardiac hypertrophy (Figures 37-41). We could therefore suggest that following similar pathways, Erk$^{Thr188}$-phosphorylation initiates tumor cell proliferation. Inversely, targeting Erk$^{Thr188}$-phosphorylation prevents nuclear translocation and, thus, cardiac hypertrophy. Similarly inhibition of Erk$^{Thr188}$-phosphorylation could retain Erk in the cytosol and inhibit proliferation. To validate these hypotheses, proliferation and apoptosis assays could be performed in breast cancer cell lines such as MCF7 or MDA cells. The relevance of Erk$^{Thr188}$-phosphorylation for these different functions could be determined using the different Erk mutants.

# CONCLUSION

Pathological cardiac hypertrophy is considered as a major risk factor of heart failure, the first cause of mortality in the Western World. Understanding the biological mechanisms involved in this detrimental pathology and translating them into pharmaceutical drugs is therefore an important challenge. Several studies have reported the relevance of Erk1/2 in hypertrophic pathways. However, unspecific blockage of the entire Raf/Mek/Erk cascade cannot be the solution. Indeed Erk1/2 are ubiquitous proteins involved in many biological and physiological events. They regulate several important cellular functions such as proliferation, cell survival and differentiation. Therefore inhibiting the whole cascade would bring severe side effects. The challenge is to understand precisely the mechanisms in order to target a precise signaling component and, thus, only the pathological effects of the cascade while preserving the cardioprotective Erk1/2 effects.

A selective inhibition of the hypertrophic function of Erk1/2 is particularly desirable in the heart since Erk1/2 also mediates protective, anti-apoptotic effects in the heart. $Erk^{Thr188}$-phosphorylation seems to be a possible candidate for such a selective interference with the Erk1/2 signaling cascade. The observation that transgenic mice overexpressing the phosphorylation deficient mutant $Erk2^{T188S}$ displayed a significantly reduced hypertrophic response and a preserved cardiac structure is the first indication for $Erk^{Thr188}$-phosphorylation as a promising selective targeting strategy. Further, *in vitro* studies revealed that interference with $Erk^{Thr188}$-phosphorylation leads to the retention of Erk in the cytosol preventing further activation of Erk nuclear target involved in cardiac hypertrophy development (Ruppert et al, 2013). These results suggest that $Erk^{Thr188}$-phosphorlyation can be a target to inhibit selective Erk functions.

Moreover, knowing that $Erk^{Thr188}$-phosphorylation is mediated by several cardiac G protein coupled receptors, such as β-adrenergic receptors or angiotensin receptors, focuses future research on a specific nod that link all these pathways together. This concept raises challenging perspectives both for common knowledge and pharmaceutical drug design.

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# CURRICULUM VITAE

**Civil Status**

| | |
|---|---|
| Name | Marie Vidal |
| Date of birth | 16th August 1985 |
| Place of birth | Dijon |
| Nationality | French |

**Education**

| | |
|---|---|
| Since September 2008 | PhD student at the Institute of Pharmacology and Toxicology, in the group of Dr. Kristina Lorenz, in Würzburg, Germany |
| June 2013 | Law degree at the International Center of Intellectual Property (CEIPI), in Strasbourg, France |
| November 2008 | Engineer degree in Chemistry |
| 2007-2008 | Student at the National School of Chemistry (ENSCM), in Montpellier, France |
| 2005-2007 | Student at the European School of Chemistry, Polymers and Materials (ECPM), in Strasbourg, France |
| 2003-2005 | Undergraduate courses to prepare the national competitive exams to enter French engineering schools, lycée Carnot, in Dijon, France |
| June 2003 | Final high school degree in science, lycée Charles de Gaulle, in Dijon, France |

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C. Ruppert, K. Deiss, S. Herrmann, M. Vidal, M. Oezkur, A. Gorski, F. Weidemann, M.J. Lohse and K. Lorenz (2013). Interference with ERK(Thr188) phosphorylation impairs pathological but not physiological cardiac hypertrophy. Proceedings of the National Academy of Sciences 110(18): 7440-5

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# ABBREVIATIONS

| | |
|---|---|
| α-MHC | α-myosin heavy chain |
| β-AR | β-adrenergic receptor |
| βark-ct | C-terminus of β-adrenergic receptor kinase |
| AC | adenylyl cyclase |
| ANP | atrial natriuretic peptide |
| BNP | B-type natriuretic peptide |
| BrdU | bromodeoxyuridine |
| CAMKII | $Ca^{2+}$/calmodulin-dependent protein kinase II |
| cAMP | cyclic adenosine 3', 5'-monophosphate |
| CMs | cardiomyocytes |
| COS-7 | monkey kidney cells |
| DMEM | Dulbecco's modified eagle medium |
| EF | ejection fraction |
| EGF | extracellular growth factor |
| FCS | fetal calf serum |
| FS | fractional shortening |
| Erk1/2 | extracellular signal-regulated kinases 1/2 |
| GDP | guanine diphosphate |
| GFP | green fluorescent protein |
| GPCR | G protein coupled receptor |
| GRK | G protein coupled receptor kinase |
| GTP | guanine triphosphate |
| HEK293 | human embryonic kidney 293 cells |
| IGF1 | insulin-like growth factor 1 |
| IVS | interventricular septum |
| JNK | c-Jun N-terminal kinase |
| KSR | kinase suppressor of Ras |
| LA | left atria |
| LTCC | L-type calcium channel |
| LV | left ventricle |
| LVID | left ventricular diameters |
| LVPW | left ventricular posterior wall |
| MAPK | mitogen activated protein kinase |

| | |
|---|---|
| MAPKK | mitogen activated protein kinase kinase |
| MAP3K | mitogen activated protein kinase kinase kinase |
| Mek1/2 | mitogen-activated protein kinase/Erk kinase 1/2 |
| MEM | minimum essential medium |
| MKPs | MAPK phosphatases |
| NFAT | nuclear factor of activated T-cells |
| NRCMs | neonatal rat cardiomyocytes |
| PCR | polymerase chain reaction |
| PI3K | phosphatidylinositol-3-kinase |
| PFA | paraformaldehyde |
| PKA | protein kinase A |
| PKC | protein kinase C |
| PLC | phospholipase C |
| PPs | phosphatases |
| PTPs | protein Tyr phosphatase |
| PTX | *pertussis toxin* |
| RKIP | Raf kinase inhibitor protein |
| RTK | receptor tyrosine kinase |
| RyRs | ryanodine receptors |
| SERCA | sarco/endoplasmic reticulum $Ca^{2+}$ ATPase |
| TAC | transverse aortic constriction |
| TEY-motif | Thr-Glu-Tyr or $T^{183}$-E-$Y^{185}$ |
| WT | wild-type |
| YFP | yellow fluorescent protein |

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