ERRα and ERRγ Target Gene Identification by Genome-Wide Location Analysis in the Mouse Heart

Catherine Rosa Dufour

Department of Biochemistry

McGill University

Montréal, Québec, Canada

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ABSTRACT

Transcriptional regulation by nuclear receptors is vital for a number of different biological processes such as cellular differentiation, embryo development and homeostasis. Recent studies on the roles of estrogen-related receptor α (ERRα) and γ (ERRγ) have demonstrated an important function of these orphan receptors in the control of cardiac energy metabolism. Chromatin immunoprecipitation (ChIP)-on-chip was optimized and then performed using chromatin from mouse hearts on a genome-wide basis to provide further insight into the roles of these ERRs in the heart. In this thesis, we report the identification of ERRα and ERRγ direct target genes in cardiac tissue implicated in various processes such as apoptosis, transcriptional regulation, signal transduction, heart development, electron transport/oxidative metabolism, as well as lipid, carbohydrate, and protein metabolism. However, one important and unexpected outcome of the experiments was the significant number of ERR target genes associated with cardiac hypertrophy in humans.

RÉSUMÉ

La régulation de la transcription par les récepteurs nucléaires est essentielle pour différents processus biologiques fondamentaux comme la différenciation cellulaire, le développement embryonnaire et l'homéostasie. Dans le contrôle du métabolisme énergétique cardiaque particulièrement, les deux récepteurs orphelins reliés à l'estrogène ERRα et ERRγ occupent une fonction importante. Pour caractériser l'action génomique globale de ces deux facteurs dans le cœur et ainsi générer de nouvelles connaissances sur leur rôle exercé, la technologie ChIP-sur-chip a premièrement été optimisée avec de la chromatine de cœurs de souris et ensuite performée. Dans cette thèse, je décris l'identification de nouveaux gènes cible directs ERRα et ERRγ, impliqués dans différents processus comme l'apoptose, la régulation de la transcription, la transduction du signal, le développement du cœur, le transport d'électron, le métabolisme oxydatif ainsi que le métabolisme des lipides, des hydrates de carbone et des protéines. L'identification de plusieurs gènes associés à l'hypertrophie cardiaque chez l'humain constitue une découverte spécialement importante.

PREFACE – CONTRIBUTION OF AUTHORS

The research conducted and presented in this thesis is entirely my own. The mouse 19K promoter microarrays for ChIP-on-chip experiments were developed and printed at the Institut de Recherches Cliniques de Montréal. This thesis was written by me with corrections by Dr. Vincent Giguère, Josée Laganière and Brian Wilson.

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ABBREVIATIONS

ADP	adenosine diphosphate
AF	activation function
Akt	serine-threonine kinase
ANT	adenine nucleotide translocase
ATP	adenosine triphosphate
Atp5b	ATP synthase, mitochondrial F1 complex, beta subunit
Atp5g3	ATP synthase, mitochondrial F0 complex, subunit 9, isoform 3
Aven	apoptosis, caspase activation inhibitor
BAX	BCL-2 associated X protein
bp	base pair
CaMK	calcium/calmodulin-dependent protein kinase
Casq2	calsequestrin 2
Cdk	cyclin-dependent kinase
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
Ckmt	creatine kinase, mitochondrial
Clybl	citrate lyase beta like
Cox	cytochrome c oxidase
CPT	carnitine palmitoyl transferase
CPVT	catecholamine-induced polymorphic ventricular tachycardia
CREG	cellular repressor of E1A-stimulated genes

Crsp3	cofactor required for Sp1 transcriptional activation, subunit 3
CTE	carboxy-terminal extension
Cycs	cytochrome c, somatic
DAG	diacylglycerol
DBD	DNA binding domain
DES	diethylstilbestrol
DGK	diacylglycerol kinase
DNA	deoxyribonucleic acid
d.p.c.	days post coitus
Eef1a2	eukaryotic translation elongation factor 1 alpha 2
Eef1b2	eukaryotic translation elongation factor 1 beta 2
Eif2b4	eukaryotic translation initiation factor 2B, subunit 4 delta
ER	estrogen receptor
ERE	estrogen response element
ERK	extracellular responsive kinase
ERR	estrogen-related receptor
ERRE	ERR response element
Esrra	Estrogen-related receptor alpha
Esrrb	Estrogen-related receptor beta
Esrrg	Estrogen-related receptor gamma
Fabp3	fatty acid binding protein 3, muscle and heart
Fh	fumarate hydratase
Gabpa	GA repeat binding protein, alpha

Got1	glutamate oxaloacetate transaminase 1, soluble
Got2	glutamate oxaloacetate transaminase 1, mitochondrial
GSK	glycogen synthase kinase
h	hour
H6pd	hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase)
HDAC	histone deacetylase
HRE	hormone response element
Htr2b/5-HT_{2B}R	5-hydroxytryptamine (serotonin) receptor 2B
JAK	janus kinase
JNK	c-Jun NH_2-terminal kinase
LBD	ligand binding domain
Ldb	LIM domain binding
LM-PCR	ligation-mediated PCR
Map3k4	mitogen activated protein kinase kinase kinase 4
MAPK	mitogen-activated protein kinase
MCAD	medium-chain acyl-coenzyme A dehydrogenase
Mdh1	malate dehydrogenase 1, NAD (soluble)
MEF2	myocyte enhancer factor 2
min	minutes
Mrpl	mitochondrial ribosomal protein L
Mrps	mitochondrial ribosomal protein S
mTOR	mammalian target of rapamycin
Myl	myosin, light polypeptide

Ndufa	NADH dehydrogenase (ubiquinone) 1 alpha
Ndufb	NADH dehydrogenase (ubiquinone) 1 beta
Ndufs	NADH dehydrogenase (ubiquinone) Fe-S protein
Ndufv	NADH dehydrogenase (ubiquinone) flavoprotein
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor-κB
NRF	nuclear respiratory factor
4-OHT	4-hydroxytamoxifen
Osbpl	oxysterol binding protein-like
Oxt	oxytocin
PA	phosphatidic acid
PCR	polymerase chain reaction
PDK	pyruvate dehydrogenase kinase
PGC	PPAR gamma coactivator
PHC	polyhomeotic-like (Drosophila)
PI3K	phosphoinositide 3-kinase
Pias3	protein inhibitor of activated STAT3
PKC	protein kinase C
Pla2g12a	phospholipase A2, group XIIA
PLC	phospholipase C
PPAR	peroxisome proliferator-activated receptor
Ppp1r	protein phosphatase 1, regulatory (inhibitor)
Pte	peroxisomal acyl-CoA thioesterase

Q-PCR	quantitative PCR
RAR	retinoic acid receptor
RT	room temperature
Sdh	succinate dehydrogenase complex
sec	seconds
Skiip	SKI interacting protein
Slc	solute carrier
Sptlc	serine palmitoyltransferase, long chain base
STAT	signal transducers and activators of transcription
TAC	transverse aortic constriction
Tcap	titin-cap
Tfam	mitochondrial transcription factor A
Txnl	thioredoxin-like

LITERATURE REVIEW

1. NUCLEAR RECEPTORS

Nuclear receptors play crucial roles in many cellular processes including energy homeostasis, differentiation and development by acting as transcription factors that can directly regulate the expression of genes by binding to target promoters (Mangelsdorf et al., 1995; Shiau et al., 2001). The nuclear receptor superfamily is comprised of the classic ligand-dependent transcription factors as well as the so-called 'orphan' nuclear receptors, named as such since no known natural ligands were associated to them at the time of their discovery or have been identified to date (Giguère, 1999).

1.1. Ligand-Activated Nuclear Receptors

The classic ligand-dependent members of the nuclear receptor superfamily were cloned on the basis that known hormones were responsible for their transcriptional activities (Giguère, 1999). A significant number of ligands have been identified and well characterized for these classical receptors such as the thyroid hormone, retinoids, steroids, and vitamin D (Mangelsdorf et al., 1995). The mechanism by which these receptors become ligand-activated has been well studied. The general consensus is that the binding of a ligand induces a conformational change within the nuclear receptor ligand binding domain (LBD) resulting in a newly exposed interface to which coactivators can subsequently bind (Glass and Rosenfeld, 2000).

1.2. Orphan Nuclear Receptors

A fair number of orphan receptors were discovered following low-stringency screenings of cDNA libraries with probes corresponding to well conserved DNA fragments from previously identified classic ligand-dependent nuclear receptors (Giguère, 1999). Although some orphan receptors lack either a transcriptional activation function 1 (AF-1) or 2 (AF-2) motif, they are nonetheless structurally similar to the classic members of the nuclear receptor superfamily in that the majority of them contain all the same functional domains (Giguère, 1999). Studies have shown that orphan nuclear receptors are able to activate gene transcription in the absence of exogenous ligand, being already in a constitutively active conformation (Chen et al., 2001).

1.3. Nuclear Receptor Structure

As shown in Figuere 1, nuclear receptors generally consist of a modulator domain located at the N-terminus followed by a DNA binding domain (DBD), a hinge region, an LBD and some have an additional C-terminal motif associated with no known function (Giguère, 1999).

The modulator domain, also known as the A/B domain, normally contains an AF-1 (Giguère, 1999; Vanacker et al., 1999). This N-terminal domain is the most variable region in primary sequence among nuclear receptors, however, A/B domains of different receptors sharing little sequence identity have been demonstrated to have similar activities (Giguère, 1999).

Nuclear receptors bind DNA through their DBDs, which is the most conserved domain (Renaud and Moras, 2000). The DBD consists of two zinc finger motifs and a

carboxy-terminal extension (CTE) which provides binding interfaces for DNA or protein binding (Giguère, 1999; Renaud and Moras, 2000). Nuclear receptors can activate gene transcription by directly binding to DNA at hormone response elements (HREs) as monomers, homodimers, or as heterodimers (Khorasanizadeh and Rastinejad, 2001). HREs contain one or two consensus core half-sites like AGGTCA, recognized by estrogen and estrogen-related receptors, that can be arranged as direct repeats or in an inverted or everted position (Glass, 1994).

Nuclear receptor DBDs and LBDs are connected by a hinge region that is highly variable in length and primary sequence (Glass, 1994).

The LBDs of nuclear receptors have multiple functions including ligand binding, receptor dimerization, nuclear localization, and transactivation activities (Giguère et al., 1986). Classical ligand-dependent receptor transactivation involves a second activation function domain, called AF-2, found within the LBD (Giguère et al., 1986).

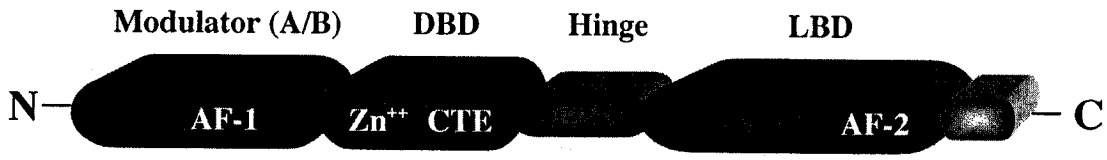


Figure 1: Nuclear receptor anatomy. Structurally, nuclear receptors typcially consist of four independent domains including a modulator or A/B domain found at the amino-terminus followed by a DBD, a hinge region and LBD. An additional carboxy-terminal domain has been found in some nuclear receptors. Two transcriptional activation function (AF-1 and -2) motifs are usually found at the amino and carboxy terminal ends, respectively. Two zinc finger motifs and a CTE are found within the DBD.

1.4. Nuclear Receptor Activity

The majority of nuclear receptors are found within the nucleus, usually bound to DNA in the absence of ligand (Tsai and O'Malley, 1994). However, some nuclear receptors are found in the cytoplasm bound to heat shock proteins, and only upon ligand binding can they switch from an inactive to an active state and translocate to the nucleus and activate gene expression (Giguère, 1999). Coregulators, being either coactivators or corepressors, can modulate nuclear receptor activity by activating or repressing gene transcription, respectively (Glass and Rosenfeld, 2000; Naar et al., 2001). In the absence of ligand, a number of nuclear receptors have been reported to act as repressors of transcription (Chen and Evans, 1995; Horlein et al., 1995). The bound repressor has been shown to be displaced upon ligand binding allowing coactivator binding (Giguère, 1999). In addition to coregulators, nuclear receptors can also control gene expression through direct binding to other transcription factors bound to DNA and can thus mediate transactivation of genes through indirect DNA binding (Giguère, 1999).

2. THE ERR ORPHAN NUCLEAR RECEPTOR FAMILY

The ERR family consists of three isoforms, α, β, and γ, all encoded by distinct genes namely, *Esrra*, *Esrrb*, and *Esrrg*, respectively, found on different chromosomes (Giguère, 1999). ERRs share 36% and 68% sequence identity with ERs within the LBD and DBD, respectively (Vanacker et al., 1999). As a result, ERRs can not bind to estrogens but can bind to estrogen response elements (EREs), like ERs, implying an overlap of ERR and ER regulatory capabilities (Vanacker et al., 1999). ERRs have been shown to bind as monomers or homodimers to the ERR-response elements (ERREs) defined as,

TNAAGGTCA, as does ERα, and as homodimers to EREs, an inverted repeat of the AGGTCA half-site separated by three nucleotides (Giguère, 1999; Giguère, 2002; Sladek et al., 1997; Vanacker et al., 1999). Although ERRs cannot bind to estrogen or other known natural ligands, all ERR isoforms have been shown to bind to the synthetic ligand, diethylstilbestrol (DES), and ERRβ and ERRγ can interact with 4-hydroxytamoxifen (4-OHT) (Coward et al., 2001; Greschik et al., 2004; Tremblay et al., 2001a; Tremblay et al., 2001b).

2.1. ERRα

The genes encoding ERRα and ERRβ, were cloned using a low stringency screening of human cDNA libraries using the region encoding the DBD of ERα as a probe (Giguère et al., 1988). ERRα expression has first been detected as early as 8.5 days post coitus (d.p.c.) during mouse development in the trophoblast, mesodermal cells of the visceral yolk sac, the primitive heart, and the neural tube (Bonnelye et al., 1997; Giguère, 1999). In the adult mouse, ERRα is widely expressed but is primarily found in tissues with a high capacity for fatty acid β-oxidation such as heart, skeletal muscle, kidney, and brown fat, implicating ERRα in the regulation of energy homeostasis (Carrier et al., 2004; Giguère et al., 1988; Ichida et al., 2002; Sladek et al., 1997; Vanacker et al., 1999). The role of ERRα as a regulator of energy metabolism is further supported by the enhanced activity of ERRα when bound by peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), an important coactivator involved in energy expenditure, mitochondrial biogenesis, gluconeogenesis and fatty acid metabolism (Ichida et al., 2002; Puigserver and Spiegelman, 2003; Sanyal et al., 2004; Schreiber et al., 2003). In

addition, ERRα expression increases during adipocyte differentiation and in response to certain physiological stimuli such as exposure to cold, fasting, and exercise (Carrier et al., 2004; Cartoni et al., 2005).

Esrra-null mice are viable, fertile, leaner than their wild-type counterparts and are resistant to high-fat diet-induced obesity (Luo et al., 2003). The reduced fat mass in the ERRα-deficient mice is linked to altered expression of genes involved in lipid metabolism and adipocyte differentiation (Luo et al., 2003). The levels of oxygen consumption, food intake and energy expenditure were found to be similar between wild-type and *Esrra* knock-out mice (Luo et al., 2003). In addition, *Esrra*-null mice have a decreased capacity for fatty acid β-oxidation which is directly coupled to oxidative phosphorylation (Carrier et al., 2004). Thus, *Esrra* knock-out studies have further supported the role of ERRα as a regulator of energy metabolism.

2.2. ERRβ

Esrrb transcript levels are first detected at 5.5 d.p.c. during embryonic development in a subset of cells within the extraembryonic ectoderm where it then becomes specifically expressed in the chorion at 7.5 d.p.c. (Luo et al., 1997; Pettersson et al., 1996). ERRβ is found in limiting amounts in adult murine heart, skeletal muscle, liver, stomach and kidney (Giguère et al., 1988; Pettersson et al., 1996). *Esrrb*-deficient mice are not viable due to fatally impaired chorion and placental formation leading to embryonic death at 9.5 d.p.c. to 10.5 d.p.c. (Luo et al., 1997). However, *Esrrb*-null mouse embryos can be rescued with wild-type tetraploid embryos, overcoming the placental defects (Mitsunaga et al., 2004).

2.3. ERRγ

Alternative *Esrrg* pre-mRNA splicing is the cause for at least 6 different transcripts that code for ERRγ isoforms 1 and 2 (Heard et al., 2000). *Esrrg* was the third member of the ERR family identified when searching for the gene responsible for Usher syndrome (Eudy et al., 1998). In humans, *Esrrg* is ubiquitously expressed in adult tissues (Eudy et al., 1998). Analysis of the crystal structure of the ERRγ LBD shows that ERRγ is in a transcriptionally active conformation and is able to bind to coactivators in the absence of ligand binding (Greschik et al., 2002; Huppunen and Aarnisalo, 2004).

3. REGULATORS OF CARDIAC ENERGY METABOLISM

PGC-1α is an important coactivator involved in the control of many aspects of cellular energy metabolism. PGC-1α is able to activate the expression of peroxisome proliferator-activated receptors (PPARs) and ERRs, important signaling pathways in cardiac energy fatty acid metabolism (Huss and Kelly, 2005).

3.1. PGC-1α

PGC-1α is a coactivator that plays a central role in mitochondrial metabolism and biogenesis (Huss and Kelly, 2005). In cultured cardiomyocytes, PGC-1α was found to increase mitochondrial number, enhance mitochondrial enzyme expression and augment fatty acid oxidation and oxidative phosphorylation (Lehman et al., 2000). PGC-1α can coactivate ERRα and PPARα, both important regulators of fatty acid oxidation (Huss and Kelly, 2005). p38 MAPK regulates PGC-1α and PPARα leading to an increase in the rate of fatty acid oxidation (Barger et al., 2001). In addition, PGC-1α is activated by

calcineurin and CaMK, proteins that are induced in the heart by exercise and activate genes in fatty acid oxidation and glucose oxidation, respectively (Handschin et al., 2003; Huss and Kelly, 2005). In contrast to PGC-1α activation, Cdk9 can suppress PGC-1α expression and its downstream targets, and Cdk9 overexpression leads to apoptotic cardiomyopathy (Sano et al., 2004). PGC-1α can activate NRFs (1 and 2) which control *Tfam* and other genes required for proper mitochondrial function (Huss and Kelly, 2005).

3.2. PPARs

The PPAR isoforms, α, β, and γ, are all implicated in cardiac energy metabolism to varying extents. PPARα is a central regulator of fatty acid metabolism in tissues with a high level of fatty acid oxidation such as the heart (Barger and Kelly, 2000; Huss and Kelly, 2004). Studies of *Ppara*-null mice have revealed a decreased expression of PPARα targets implicated in fatty acid uptake, transport and oxidation resulting in intracellular lipid accumulation (Campbell et al., 2002; Djouadi et al., 1998; Lee et al., 1995; Leone et al., 1999). On the other hand, mouse cardiac PPARα overexpression leads to cardiomyopathy demonstrated by hypertrophy, cardiac dysfunction and fetal gene activation (Finck et al., 2003; Finck et al., 2002; Hopkins et al., 2003). PPARβ was found to be involved in controlling the expression of fatty acid oxidation enzymes and found to enhance palmitate oxidation levels in both neonatal and adult cardiomyocytes in response to selective ligand-mediated activation similar to the efficacy of PPARα-selective ligands (Cheng et al., 2004; Dressel et al., 2003; Gilde et al., 2003). PPARγ plays a role in lipid storage particularly in adipose tissue but to a lower extent in the heart (Huss and Kelly, 2004). Like PPARα and PPARβ, PPARγ is activated by circulating

fatty acids and its regulation of lipid storage indirectly affects the activities of the other PPAR isoforms as well as insulin sensitivity in the heart (Huss and Kelly, 2004).

3.3. ERRα

Cardiac levels of ERRα increase significantly following birth in parallel with the switch from glucose to fatty acids as the primary source of energy as well as upregulation of PPARα and PGC-1α expression and enzymes involved in fatty acid uptake and β-oxidation (Huss et al., 2002). Treatment of cardiac muscle cells with an ERRα inhibitor has supported a crucial role for ERRα in PGC-1α mediated regulation of oxidative phosphorylation and mitochondrial respiration (Mootha et al., 2004). This is demonstrated by the ability of ERRα to directly transactivate *Ppara* and *Gapba* gene expression, known PGC-1α targets (Huss et al., 2004; Mootha et al., 2004).

3.4. ERRγ

Both ERRγ and PGC-1α are more highly expressed in hearts of *Esrra*-null mice (Huss and Kelly, 2004). This discovery and the fact that ERRγ can mediate PGC-1α effects, underlines a role of ERRγ in the transcriptional control of fatty acid metabolic genes in cardiac tissue (Huss and Kelly, 2004; Huss et al., 2002).

4. CHIP-ON-CHIP

ChIP is a relatively new technique constantly being modified for different purposes such as to study histone modifications and chromatin remodeling (Fournier et al., 2002; Sacks and Shi, 2000) but has been more commonly used to identify transcription factor binding sites or to validate potential target promoters (Barski and Frenkel, 2004; Hug et al., 2004; Wells and Farnham, 2002). ChIP essentially promotes a quantitative assessment of the relative binding of a specific protein to DNA in cultured cells or tissues (Geisberg and Struhl, 2004a; Geisberg and Struhl, 2004b). On the down side, standard ChIP can not address whether or not two different proteins simultaneously occupy a specified genomic region, and so, sequential ChIP may be performed whereby two consecutive immunoprecipitations are done with different antibodies specific to the two proteins in question (Geisberg and Struhl, 2004a; Geisberg and Struhl, 2004b). In addition, the precise nucleotide sequence to which the target proteins are found bound is not determined from ChIP alone but may be inferred or predicted using previous knowledge of known consensus binding sites (Kang et al., 2002). However, target-bound DNA regions can be discovered with no prior need of suspected binding sites and, conventional ChIP, unlike electromobility shift assays and promoter-reporter assays, can be used to study protein-DNA interactions *in vivo* (Barski and Frenkel, 2004; Kazi et al., 2005).

ChIP-based approaches include ChIP-cloning, ChIP-Display, and ChIP-on-chip (Barski and Frenkel, 2004; Kurdistani et al., 2002; Weinmann and Farnham, 2002). For our purposes, we wanted to use ChIP-on-chip, a technique that couples standard ChIP to a DNA microarray to identify specific transcription factor bound target promoters on a genome-wide scale (Geisberg and Struhl, 2004b). More specifically, we were interested

in optimizing ChIP-on-chip in mouse tissues to discover novel ERRα and ERRγ target genes in a tissue-specific manner and on a genome-wide basis. This method entails first crosslinking protein to chromatin regions followed by sonication and then selective enrichment of DNA fragments bound to the protein of interest through immunoprecipitation with a specific antibody. Subsequently, the protein-DNA crosslinks are reversed, amplified by ligation-mediated PCR and enriched Cy5-labeled DNA fragments are hybridized along with Cy3-labeled non-enriched input to a promoter microarray containing portions of 19,000 mouse genes. Hybridized spots with a stronger signal for the enriched DNA fragments relative to the control input sample suggests a positive direct target gene. Of note, the custom promoter microarrays used in our study contained genomic regions spanning from 800 base pairs upstream and 200 base pairs downstream of promoter transcriptional start sites. Despite the fact that some transcription factor response elements are found at more distal regions, the majority of the known transcription factor binding sites have been located proximal to the start-site (Odom et al., 2004).

GOAL OF THIS STUDY

Studies of the orphan nuclear receptors, ERRα and ERRγ, in the control of energy homeostasis has been soaring due to the recent discovery of the ability of these receptors to interact with the coactivator, PGC-1α, which plays a crucial role in multiple aspects of energy metabolism (Huss and Kelly, 2005; Huss et al., 2002; Ichida et al., 2002; Laganiere et al., 2004; Mootha et al., 2004; Schreiber et al., 2004). These orphan receptors are found expressed to a larger extent in tissues with high levels of mitochondrial fatty acid metabolism including the heart (Giguère et al., 1988; Heard et al., 2000; Sladek et al., 1997). To date, a relatively limited amount of ERRα cardiac target genes have been identified and to a lesser extent for ERRγ. The cardiac ERRα targets discovered have been implicated in fatty acid uptake, β-oxidation, in the respiratory chain as well as in oxidative phosphorylation (Huss and Kelly, 2004).

ChIP-on-chip is a relatively novel technique that can be applied for the identification of transcription factor target genes on a genome-wide scale analysis. The aim of this thesis was to optimize ChIP-on-chip in mouse heart as a means to discover novel ERRα and ERRγ target genes to provide further insight into their roles in cardiac energy metabolism.

MATERIALS AND METHODS

Animals

Adult male C57BL/6J mice aged 2-3 months were housed and fed standard chow in the animal facility at the McGill University Health Centre.

ChIP Assays for Target Validation

ChIP assays were performed on hearts obtained from adult male mice of 2-3 months old. Specifically, hearts were isolated, weighed and homogenized for a few seconds (until no tissue clumps) in 14 ml culture tubes containing 5 ml of cold PBS using a Brinkmann polytron homogenizer. The samples were centrifuged at 2000 rpm for 2 min at 4°C and the cell pellets were resuspended in cell lysis buffer (5 mM HEPES pH 8, 85 mM KCl, 0.5% NP-40) containing a protease inhibitor cocktail (Roche) and incubated at 4°C for 15 min with rotation. Following centrifugation at 2000 rpm for 2 min at 4°C, the nuclei pellets were resuspended in PBS containing formaldehyde (1% final) and DNA-protein crosslinking was allowed to occur at room temperature for 20 min with rotation. Samples were then centrifuged at 2000 rpm for 2 min at 4°C and the nuclei pellets were washed twice with 10 ml cold PBS and stored at –80°C. Nuclear pellets were thawed and resuspended in approximately 3 ml nuclei lysis buffer (50 mM Tris-HCl, pH 8.1, 10 mM EDTA, 1% SDS) supplemented with protease inhibitors (Roche) per gram of initial heart weight. The resuspended nuclear pellets were pooled and sonicated on ice in 50 ml Falcon tubes at power 10 for 30 sec pulses using a VirSonic 100 (Virtis) sonicator at 30

sec intervals to prevent the samples from heating too much. A total of 20 min of sonication was necessary for samples of about 7 ml of resuspend nuclear pellets. Sonicated material was centrifuged at maximum speed for 15 min at 4°C in 1.5 ml eppendorfs. For standard ChIP experiments, 10% input samples were prepared by incubating sonicated material corresponding to 0. 015 g of starting cardiac weight and eluted in 150 µl elution buffer (1% SDS, 0.1 M $NaHCO_3$) at 65°C for at least 6 h. Thereafter, the 10% inputs were purified using the QIAquick Spin Kit (Qiagen) and eluted from the column with 30 µl of elution buffer provided in the kit. For antibody-enriched and no-antibody control samples, chromatin corresponding to 0.15 g of initial heart mass was diluted in 2.5X ChIP dilution buffer (0.5% Triton X-100, 2 mM EDTA, 100 mM NaCl, 20 mM Tris-HCl, pH 8.1) in 1.5 ml eppendorfs and pre-cleared using 50 µl of a 50% slurry of salmon sperm DNA/protein A beads (Upstate) for 1 h at 4°C. Pre-cleared chromatin was either immunoprecipitated (target enriched material) or not (no antibody control) overnight with an anti-hERRα polyclonal antibody developed in our laboratory (specific for both human and mouse ERRα) or with a purified murine specific anti-mERRγ polyclonal antibody (gift from Ronald M. Evans, La Jolla, Ca) with subsequent addition of 50 µl of a 50% slurry of salmon sperm DNA/protein A beads for 2 h at 4°C. The beads were washed sequentially in 1 ml for 10 min at 4°C with three different buffers requiring centrifugation at 4000 rpm at 4°C after each wash. Initially, buffer I (1% Triton X-100, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, pH 8.0, 20 mM Tris-HCl, pH 8.1) was used, then buffer II (1% Triton X-100, 0.1% SDS, 500 mM NaCl, 2 mM EDTA, pH 8.0, 20 mM Tris-HCl, pH 8.1) and finally with buffer III (1% NP-40, 0.25 mM LiCl, 1% Na-deoxycholate, 1 mM EDTA, pH 8.0, 10 mM Tris-HCl, pH 8.1).

The beads were next washed briefly with TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0) and eluted in 150 μl elution buffer (1% SDS, 0.1 M $NaHCO_3$) at 65°C for at least 6 h. The eluate was purified using the QIAquick Spin Kit (Qiagen) and eluted from the column with 30 μl of elution buffer provided in the kit.

ChIP Assays for Genome-Wide Location Analysis

The 10% inputs, immunoprecipitated and no-antibody control samples were prepared as described in the standard ChIP protocol above for target validation with the following modifications: for the 10% inputs, sonicated material corresponding to 0.15 g of starting cardiac weight was incubated at 65°C for at least 6 h in SDS (1% final) and $NaHCO_3$ (0.1M final) and purified from QIAquick Spin columns with 2 consecutive 30 μl elutions with elution buffer supplied in the kit; 15 mL Falcon tubes were used for enriched and no-antibody control samples and sonicated material corresponding to 1.5 g initial heart mass was used; a volume of 400 μl of a 50% slurry of salmon sperm DNA/protein A beads was used for both a 2 h pre-clear and 3 h incubation prior to sequential washing with 10 ml volumes of wash buffers; samples were eluted in 1 ml elution buffer at 65°C for at least 6 h and purified from QIAquick Spin columns with 2 consecutive 30 μl elutions with elution buffer supplied in the kit.

Genome-Wide Location Analysis

Triplicate ERRα and ERRγ genome-wide location analysis experiments were performed. Sample preparation of enriched and input control material for hybridizations to mouse promoter microarrays involves ligation-mediated PCR (LM-PCR) and Cy-dye labeling.

First, the 10% inputs were diluted with H_2O to a total of 55 µl to the percentage required for the input samples to read at the same Q-PCR cycle as that for the enriched samples using control primers. Then, 55 µl of non-diluted purified enriched material and diluted control input samples were separately added to a 55 µl mixture containing 11 µl NEB buffer 2 (10X), 0.5 µl BSA (10 mg/ml), 1 µl 10 mM dNTP mix, 0.2 µl T4 DNA polymerase (3U/µl) and 42.3 µl of H_2O. The mixtures were incubated at 12°C for 20 min followed by addition of 12 µl of a solution containing 11.5 µl 3 M NaOAc, pH 5.2 and 0.5 µl glycogen (20 mg/ml). The samples were vortexed briefly and 120 µl of phenol/chloroform/isoamyl was added. The samples were vortexed again and centrifuged for 5 min at 13000 rpm at RT. The upper phase was transferred to a new tube followed by addition of 2 volumes of cold ethanol. The samples were vortexed and precipitated at –80°C for 1 h. The precipitated material was centrifuged at 13000 rpm, 4°C for 30 min and the pellets were air-dried. The pellets were then resuspended with a 20 µl mixture of 2 µl 10X T4 DNA ligase buffer, 6.5 µl annealed linkers (15 µM) (Ren et al., 2000), 0.5 µl 0.1 M ATP, 1 µl T4 DNA ligase (400U/µl) and 10 µl H_2O. The resuspended samples were incubated at 16°C overnight. The next day, a 20 µl solution containing 13.5 µl H_2O, 4 µl 10X Thermopol buffer, 1.25 µl 10 mM dNTP mix and 1.25 µl of 40 uM oligo oJW102 (Ren et al., 2000) was added. The samples were initiated to an LM-PCR program consisting of 4 min at 55°C, 3 min at 72°C, 2 min at 95°C, 28 cycles of 30 sec at 95°C, 30 sec at 60°C and 1 min at 72°C, followed by 5 min at 72°C and kept at 4°C until ready. The PCR program was paused once step 1 reached 2 min at 55°C, then a 10 µl solution comprised of 8 µl of H_2O, 1 µl of 10X Thermopol buffer and 1 µl TAQ (5U/µl, Invitrogen) was added and the PCR run was continued. The LM-PCR samples were

purified by QIAquick Spin kit and eluted twice with 30 µl of elution buffer provided in the kit. Purified enriched and input control samples were labeled with Cy5 and Cy3 dyes (Invitrogen), respectively. For each ChIP-on-chip sample, 1 µg purified LM-PCR'd sample was placed into two 1.5 ml eppendorfs (2µg each of enriched and control input samples needed). Next, 20 µl 2.5X Random Primers (BioPrime Kit) was added and the samples were incubated at 95°C for 5 min. The samples were cooled on ice and 5 µl dUTP was added. Then, 1 µl of Cy5 or Cy3 was added with subsequent addition of 1 µl Klenow to each tube. The samples were incubated at 37°C for 2 h covered with aluminum foil. The samples were then purified using the Invitrogen BioPrime kit and 2 µg of each sample was eluted with at total of 100 µl. To the purified samples, 1/10 volume 3M NaOAc, pH 5.2 and 2.5X volume of cold ethanol was added and the samples were precipitated at -80°C for 2 h. Subsequently, the samples were centrifuged at 4°C for 30 min at maximum speed and the pellets were washed with 70% ethanol, air-dried and stored at -20°C ready for hybridization.

Hybridizations of Cy5-labeled ERRα or ERRγ enriched material against Cy3-labeled control input DNA to mouse 19K promoter microarrays were performed at the Institut de recherches cliniques de Montréal (IRCM). First, microarray slides containing 19000 promoter regions spanning from 800 bp upstream to 200 bp downstream of promoter transcriptional start sites were blocked for 20 min in a 250 ml solution containing 4 g succinic anhydride, 239 ml 1-methyl-2-pyrrolidinone and 11 ml boric acid, pH 8. The slides were then emerged first briefly then for 20 sec into a dish containing 0.2% SDS solution, followed by 20 sec in RT water, 2 min in 95°C water and finally for a few seconds in RT water prior to centrifugation at 1500 rpm for 3 min. Cy5 labeled

enriched material was resuspended in 5 μl H_2O and combined with Cy3 labeled non-enriched material. Then, 20 μl human Cot-1 DNA (1 mg/ml) and 5 μl yeast tRNA (8 mg/ml) were added to the samples, mixed and then dried using a speed vac. The dried samples were resuspended in 50 μl hybridization buffer prepared by mixing 125 μl formamide (25% final), 125 μl 20X SSC (5X final), 5 μl 10% SDS (0.1% final), 100 μl BSA (20% final), and 145 μl H_2O. The samples were incubated at 95°C for 5 min and kept on a 50°C hot plate after a quick spin. About 47-48 μl from each sample was transferred onto a blocked mouse 19K promoter microarray slide, placed into a hybridization chamber and incubated at 50°C for at least 20 h. The microarrays were then washed consecutively with three different washing buffers with agitation. First, the arrays were washed for 15 min in Wash I solution (2X SSC, 0.1% SDS), twice for 2 min in Wash II solution (0.1X SSC, 0.1% SDS) and twice for 1 min in Wash III solution (0.1X SSC). The slides were then dried by centrifugation at 1500 rpm for 3 min, scanned and a macro was run to calculate p-values and binding ratios for the hybridized promoters on the mouse microarrays.

Quantitative Real-Time PCR

To assess the enrichment of ERRα or ERRγ at specific promoters, quantitative PCR (Q-PCR) was performed using mouse heart starndard ChIP samples. Briefly, 6.25 μl SYBR PCR Master Mix (Qiagen), 4.75 μl H_2O, 1 μl of enriched or non-enriched ChIP sample, and 0.5 μl each of the forward and reverse primers (10 μM) specific for a certain promoter was prepared per PCR reaction. Quantitative PCR was performed on a Roche LightCycler instrument with a program consisting of 15min at 95°C followed by 45

cycles of 15 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C with a transition temperature of 20°C/sec. ERR promoter occupancy was quantified using the following formula:

$$2^{(\text{Q-PCR cycle of no-antibody control DNA - Q-PCR cycle of enriched DNA})}.$$

Enrichment of DNA fragments was normalized against an amplified control region using the control primers, located approximately 4 kb upstream of the ERRα transcriptional start site. Table 1 shows the specific mouse primers designed and used for ChIP quantitative PCR analysis.

Functional Classification of Target Genes

Cellular functional classifications were based on GO annotations (http://fatigo.org/) and NCBI gene descriptions. In incidences where individual loci could be assigned to two distinct genes, both genes were included in the functional analysis.

RESULTS

Mouse Heart ERRα and ERRγ Target Genes Revealed Through ChIP-on-chip

ChIP-on-chip experiments couple standard ChIP with DNA microarray technology. ChIP-on-chip has been demonstrated as a useful technique for the identification of direct target genes bound by a certain transcription factor *in vivo* on a genome-wide scale location analysis (Geisberg and Struhl, 2004b). For our purposes, standard ChIP samples containing either ERRα or ERRγ antibody-enriched DNA fragments were hybridized along with control input samples on mouse 19K promoter microarrays to identify specific DNA regions recognized by these transcription factors. In the laboratory, ChIP was first optimized on different mouse tissues including brain, heart, kidney, liver and muscle. However, this thesis will focus on results concerning ChIP-on-chip experiments in murine hearts using both ERRα and ERRγ antibodies. Figure 2 graphically outlines the main steps involved in the ChIP-on-chip experiments performed on hearts obtained from adult male mice in a C57BL/6J background. Specifically, 51 mouse hearts were isolated, briefly homogenized with a polytron homogenizer, cells were lysed then treated with formaldehyde to cross-link protein bound to genomic DNA. Pooled cross-linked cardiac cells were subsequently sonicated and 1.5 g of initial cardiac mass was used for independent immunoprecipitations with polyclonal antibodies specific for either ERRα or ERRγ. Cy5-labeled ERRα or ERRγ enriched DNA fragments were hybridized along with Cy3-labeled control input DNA to mouse 19K promoter microarrays. Triplicate location analyses using antibodies against ERRα and ERRγ resulted in 194 and 229 target genes, respectively, at a cutoff of p-value ≤ 0.01 (Figure 3A, B and Figure 4). Interestingly, of

the ERRα and ERRγ target promoters obtained, 88 promoters were found to be recognized by both antibodies (Figure 4). Thus, approximately 45% of the ERRα targets found are also bound by ERRγ and about 38% of the ERRγ targets discovered are also occupied by ERRα. This significant overlap of ERRα and ERRγ target genes is not surprising since the two transcription factors are part of the same ERR orphan nuclear receptor subfamily and have significant sequence identity within their DNA binding domains (Giguère, 2002; Hentschke et al., 2002). When obtaining a list of promoters from a ChIP-on-chip analysis, it is necessary to validate a subset of the targets obtained with increasing p-value in order to evaluate the confidence of finding a true-positive target linked with an associated p-value from the experiment. To validate the ERRα and ERRγ ChIP-on-chip results, a number of randomly chosen genes were selected from the list of genes obtained ranging from targets with the lowest p-values to those with a p-value of ≤ 0.01. More precisely, genes were selected from three different ranges of p-values: ≤ 0.001, 0.001 to ≤ 0.005, and 0.005 to ≤ 0.01. Specific primer sets were designed for each of the selected genes, as shown in Table 1, and standard ChIP assays and quantitative PCR were performed using murine hearts to confirm ERRα and/or ERRγ occupancy at these target promoters. For ERRα and ERRγ target validation, at least 9 and 8 genes, respectively, were tested in each of the three ranges of p-values (Table 4 and Table 5). In general, genes assigned with the lowest p-values are those more likely to be true-positive targets. Surprisingly, all of the genes tested were found to be true ERRα and/or ERRγ targets. This suggests that the ERRα and ERRγ targets obtained at a cutoff of p-value ≤ 0.01 are likely to be mostly, if not all, true-positives. Thus, further target validation is necessary with genes associated with p-values > 0.01 to determine at what p-

value the likelihood of finding a true-positive falls under an 85-95% chance. Finding the cutoff of target genes with a confidence of true-positive targets of at least 85-95% will expand the list of ERRα and ERRγ targets, despite the incorporation of false-positive targets, better representing their cardiac physiological roles through their vast transcriptional capabilities. Also, identifying previously discovered ERRα or ERRγ targets not included in the list would further support the need for additional validation. In the absence of further validation, this thesis primarily focuses on ERRα and ERRγ target genes obtained at a threshold of p-value ≤ 0.01. With our threshold criteria, 158 and 180 ERRα and ERRγ target promoters, respectively, discovered were known genes and could be assigned with GO functions and NCBI gene descriptions (Table 2 and Figure 5A, B). The remaining 36 and 49 target genes of ERRα and ERRγ, respectively, with no assigned function are listed in Table 3. ERRα was found to have more direct targets involved in lipid metabolism, transcriptional regulation, signal transduction, and heart development relative to ERRγ, whereas, the ERRγ ChIP-on-chip experiments had revealed a larger number of ERRγ target genes implicated in apoptosis, electron transport/oxidative phosphorylation and protein modification/metabolism compared to ERRα. The two ERR isoforms had a similar number, although not necessarily the same, target genes involved in cellular functions such as amino acid and carbohydrate metabolism, cell adhesion, cell motility, cell proliferation, chromosome biogenesis, cytoskeleton, defense response, energy transfer, muscle development, immune response, nucleic acid metabolism, and organogenesis.

ERRα and ERRγ Control of Energy Metabolism

ERRα and ERRγ are known to be found primarily expressed in tissues with high mitochondrial oxidative potential such as the heart and skeletal muscle (Giguère et al., 1988; Heard et al., 2000; Sladek et al., 1997). Much evidence, more so for ERRα, has supported an important role of these ERR isoforms as regulators of energy homeostasis including their ability to mediate PGC-1α effects, a key metabolic coactivator in the regulation of energy metabolism such as mitochondrial biogenesis and oxidation (Puigserver and Spiegelman, 2003). ERRα and ERRγ ChIP-on-chip experiments have provided further insight into the roles of these transcription factors in energy metabolism. Of all the functions that were assigned to target genes, 73 are common to both ERRα and ERRγ. The greatest overlap in ERRα and ERRγ target genes are implicated in electron transport and oxidative phosphorylation including *Atp5b*, *Atp5g3*, *Cox6c*, *Cycs*, *Fhl*, *Ndufa9*, *Ndufb4*, *Ndufb5*, *Ndufs7*, *Sdha*, *Sdhb* and *Txnl2* (Figure 5C and Table 2). In addition, genes encoding the malate-aspartate shuttling proteins, GOT1 and GOT2, were found to be regulated by both ERRs and ERRγ, respectively. These target genes are important in oxidative phosphorylation such that decreased expression of these proteins results in impaired cardiac glycolysis and decreased mitochondrial energy metabolism (Fountoulakis et al., 2005). ERRα and/or ERRγ targets were also found in specific biological processes such as fatty acid uptake (*Fabp3*, *Osbpl3*), fatty acid transport (*Slc25a29*), β-oxidation (*Acadm*, *Sptlc2*, *Pte2a*), glucose transport (*Slc2a12*) and glucose metabolism (*H6pd*, *Pdk4*). Expression of pyruvate dehydrogenase kinase 4 (PDK4) leads to increased fatty acid oxidation through inhibition of the pyruvate dehydrogenase complex impairing glucose oxidation (Huss and Kelly, 2004). *Pdk4* was found as an

ERRγ target but was associated with a p-value slightly greater than 0.01 from the ERRα ChIP-on-chip experiments, therefore not making the threshold criteria. However, ChIP analysis (data not shown) and a recent publication confirm that *Pdk4* is indeed an ERRα target (Wende et al., 2005). In addition, ERRα has previously been demonstrated to mediate PGC-1α activation of the nuclear respiratory factor (NRF) pathway through regulation of the *Gapba* gene, which encodes for the NRF-2 subunit (Mootha et al., 2004). NRFs are known to regulate transcription of the mitochondrial transcription factor A (*Tfam*), thus, playing an important role in mitochondrial biogenesis (Huss and Kelly, 2005). Location analysis proved *Gapba* to be a direct target gene of both ERRα and ERRγ. Another interesting ERRα and ERRγ target is *Clybl*, since inhibition of the encoded enzyme may increase aerobic endurance by favoring gluconeogenesis (McCarty, 1995). In contrast, absence of the ERRα and ERRγ target *Slc25a4*, better known as *ANT-1*, results in impaired oxidative phosphorylation and increased lactic acid production leading to severe exercise intolerance (Graham et al., 1997). Another important gene in the control of energy homeostasis is the ERRα and ERRγ target creatine kinase mitochondrial 2 (*Ckmt2*). CKMT2 acts by phosphorylating creatine to phosphocreatine, being the main energy store in the heart (Huss and Kelly, 2005; Momken et al., 2005). Furthermore, mouse knock-out studies on the ERRγ target gene, *Ppp1r3a*, encoding a muscle-specific subunit of protein phosphatase 1, has revealed that the absence of this gene leads to significantly impaired glycogen accumulation (Suzuki et al., 2001). Collectively, these findings of previously identified but mostly novel ERRα and ERRγ targets not only confirm but enhance the importance of the role of these transcriptional regulators in mitochondrial biogenesis and energy homeostasis.

ERRα and ERRγ Targets Linked to Cardiac Hypertrophy and Cardiomyopathy

Cardiac hypertrophy is linked with a decreased reliance on fatty acid oxidation and an increase in carbohydrate energy expenditure (Taegtmeyer et al., 2002). An unexpected outcome from functional analyses and literature review of target genes obtained from ERRα and ERRγ genome-wide location experiments, was a small but significant number of genes associated with cardiac hypertrophy, as shown in Table 6. For example, deficiency in the protein encoded by the previously mentioned ERRα and ERRγ target *ANT-1* leads to cardiac hypertrophy and mitochondrial proliferation in mice (Graham et al., 1997). ANT-1 exchanges mitochondrial matrix ATP for cytosolic ADP and is thus a key factor in oxidative phosphorylation providing mitochondrial energy to the cytosol (Brown and Wallace, 1994; Klingenberg, 1992). Another discovered cardiac hypertrophy associated gene, aside from its role in energy metabolism, is the ERRα and ERRγ target, *Ckmt2*. Impaired coupling of creatine kinase and ANT-1 activities has been shown to be associated with cardiac hypertrophy and chamber dilation (Fountoulakis et al., 2005; Nahrendorf et al., 2005). Expression of the ERRγ target, *Mdh1*, plays a role in regulating both the tricarboxylic acid cycle and contractile function (Lo et al., 2005). Hearts of patients with dilated cardiomyopathy were found to overexpress MDH1 supporting the increased reliance on glucose oxidation during cardiac hypertrophy (Lo et al., 2005). Although *Rara,* also known as *Rarα,* did not show up as an ERRα target having a p-value greater than 0.01, it is indeed regulated by ERRα in addition to ERRγ supported by standard ChIP analysis and quantitative PCR (data not shown). RARα has an established role in heart development (Lee et al., 1997) and hearts overexpressing RARα result in dilated cardiomyopathy, eventually leading to congestive cardiac failure (Colbert et al.,

1997; Kastner et al., 1997). Coincidently, oxytocin, encoded by the *Oxt* gene, another target of ERRα and ERRγ, is a female reproductive hormone also found to play a role in heart development (Paquin et al., 2002). Interestingly, retinoic acid was found to activate oxytocin expression in the fetal heart (Paquin et al., 2002). Thus, it can be stipulated that hearts overexpressing OXT, similarly to overexpression of RARα, may lead to cardiomyopathy. Despite the fact that *Fabp3* is not shown as an ERRα target in Table 2, it is a known direct ERRα target (Huss and Kelly, 2004). High serum levels of FABP3, a long-chain fatty acid binding protein, is a marker of ongoing myocardial damage in patients with hypertrophic cardiomyopathy and chronic heart failure (Binas et al., 2003; Morioka et al., 2005; Setsuta et al., 2002). Gp130 receptor signaling leads to JAK activation with subsequent activation of STAT3 which has been shown to activate hypertrophic response gene expression (Frey and Olson, 2003). ChIP-on-chip revealed the gene encoding for the protein inhibitor of activated STAT3, Pias3, as a target of ERRα. In addition, a number of gene mutations were associated with cardiac hypertrophy, cardiomyopathy or heart failure. For instance, a missense mutation found in the ERRα and ERRγ target, *Casq2,* is associated with catecholamine-induced polymorphic ventricular tachycardia (CPVT) which is linked to sudden cardiac death in response to stress (Lahat et al., 2001a; Lahat et al., 2001b; Postma et al., 2002). Also, mutations in the ERRα and ERRγ target *Myl3*, encoding a muscle protein, has been linked to hypertrophic cardiomyopathy (Morita et al., 2005). Furthermore, mutations in the ERRγ target genes, *Tcap* and *Ldb3*, encoding muscle Z-disc proteins, may lead to dilated or hypertrophic cardiomyopathy predisposing an individual to heart failure (Arimura et al., 2004; Morita et al., 2005; Vatta et al., 2003). Moreover, mutations in the

ERRγ target *Ndufs2*, a gene involved in the respiratory chain, is linked with hypertrophic cardiomyopathy (Goffart et al., 2004). Studies of cardiomyocytes overexpressing the encoded protein from the ERRα target *Phc1*, has shown that enhanced cardiac levels of PHC1 leads to dilated cardiomyopathy associated with cardiac muscle cell death, myofibril defects and severe cardiac failure (Koga et al., 2002). The ERRα target, 5-hydroxytryptamine (serotonin) receptor 2B (*Htr2b*), also referred to as *5-HT_{2B}R*, was shown to lead to hypertrophic cardiomyopathy and dilated cardiomyopathy as a result of mouse cardiac overexpression and gene ablation, respectively (Nebigil and Maroteaux, 2003). Interestingly, 5-HT_{2B}R crosstalk with 5-$HT_{1B/1D}$ receptor subtypes occurs through phospholipase A_2 activation and the gene for Pla2g12a, a subtype of phospholipase A_2, is also an ERRα target identified (Nebigil et al., 2001). Studies have shown that Gq-coupled 5-HT_{2B}R activation in turn activates phosphoinositide 3-kinase/serine-threonine kinase (PI3K/Akt) and extracellular responsive kinase (ERK) subsequently resulting in ablation of ANT-1 and BAX expression, respectively (Nebigil and Maroteaux, 2003). The ERRγ target *Trp53* encodes a pro-apoptotic protein that can lead to cardiomyocyte apoptosis through BAX activation (Perik et al., 2005). Activation of the intrinsic death pathway leads to increased mitochondrial membrane permeability which is maintained by a number of proteins including ANT-1 and BAX (Baines and Molkentin, 2005; Nebigil and Maroteaux, 2003). Thus, in the absence of 5-HT_{2B}R, the mitochondrial membrane permeability pore is no longer maintained resulting in cytochrome c release and eventually in dilated cardiomyopathy (Nebigil and Maroteaux, 2003). On the other hand, overexpresssion of 5-HT_{2B}R results in ANT-1 downregulation associated with increased mitochondrial respiratory chain function and oxidative phosphorylation leading to

hypertrophic cardiomyopathy (Nebigil et al., 2001; Nebigil et al., 2003). Figure 6 depicts ERRα and/or ERRγ regulated target genes in signaling pathways associated with cardiac hypertrophy. The hypertrophic program can be activated by binding of the neurohormone, serotonin, to its receptor Htr2b (Nebigil and Maroteaux, 2003). This in turn leads to activation of ERK and PI3K/Akt pathways aside from increased Sp1 expression and phospholipase C (PLC) mediated diacylglycerol (DAG) production and nuclear factor of activated T cells (NFAT) activation (Dorn and Force, 2005; Huss and Kelly, 2005; Nebigil and Maroteaux, 2003). ERK signaling can impair BAX expression, a pro-apoptotic regulator downstream of Trp53 capable of inducing apoptosis through caspase activation (Nebigil and Maroteaux, 2003; Perik et al., 2005). Location analysis revealed that ERRα regulates the gene encoding an apoptosis caspase activation inhibitor referred to as AVEN. Activation of PI3K/Akt signaling leads to NF-κB activation in turn inhibiting both PDK4 and ANT-1, resulting in impaired glucose oxidation and increased mitochondrial permeability, respectively (Nebigil and Maroteaux, 2003; Planavila et al., 2005). More specifically, NF-κB activation leads to enhanced NF-κB binding to PPARβ/δ, preventing the PPAR isoforms to activate the expression of PDK4 (Planavila et al., 2005). ERRα can regulate NF-κB activity through expression of its target, *Skiip*, which has been shown to be part of a corepressor complex that can repress the transactivation of genes including *NF-κB* (Zhou et al., 2000). The transcription factor, Sp1, capable of repressing the expression of important fatty acid oxidation genes is induced in the hypertrophied mouse heart (Huss and Kelly, 2005; Sack et al., 1997). Thus, Sp1 is an important target in cardiac hypertrophy. To this end, the gene encoding CRSP3, an activator of Sp1 activity was found to be an ERRα and ERRγ target. A likely

Sp1 target is *Ppara*, a key regulator of fatty acid metabolism, since PPARα is down-regulated during cardiac hypertrophy resulting in decreased fatty acid oxidation and an increased reliance on glucose and lactate as substrates for energy production (Barger et al., 2000). Together, increased Sp1 expression and PI3K/Akt signaling play an important part in the observed decrease in fatty acid oxidation and increased reliance on carbohydrate metabolism during cardiac hypertrophy (Huss and Kelly, 2005; Planavila et al., 2005). PI3K/Akt signaling also leads to the activation of mTOR, which activates protein synthesis, another feature of cardiac hypertrophy (Dorn and Force, 2005). GSK3 can act to inhibit protein synthesis through inhibition of factors for translation such as the protein translation initiation factor Eif2b (Dorn and Force, 2005). Interestingly, the ERRγ target, *Eif2b4*, encodes a subunit of Elf2b. A number of ERRα and/or ERRγ targets involved in protein synthesis are shown in Figure 6, including *Mrps18b*, *Mrp134*, *Mrp147*, *Eef1a2* and *Eef1b2*. GSK3 can also prevent cardiac hypertrophy through inhibition of hypertrophic response gene expression by phosphorylating calcineurin and NFAT (Hardt and Sadoshima, 2002; McKinsey and Olson, 2005). PLC signaling leads to an augmentation in calcium levels which with calmodulin activates calcineurin which in turn dephosphorylates NFAT subsequently leading to NFAT activation of gene transcription (Dorn and Force, 2005; McKinsey and Olson, 2005). GSK3 activity can be inhibited through activation of the ERK pathway and DAG activation of PKCs, favoring the development of cardiac hypertrophy (Dorn and Force, 2005). PKCs were demonstrated to control cardiac muscle contractility and hypertrophy in cultured cardiomyocytes (Dorn and Mochly-Rosen, 2002). Specifically, PKCα and PKCβ upregulation and PKCε activation have been found associated with either myocardial

hypertrophy or heart failure (Bowling et al., 1999; Wang et al., 1999). The ERRγ target, diacylglycerol kinase theta (*Dgkθ*) encodes a kinase found to negatively regulate DAG by phosphorylating it to phosphatidic acid (PA) (van Baal et al., 2005). Interestingly, a study demonstrated that DGKθ was induced by PKC isoforms forming a negative feedback loop as shown in Figure 6 (van Baal et al., 2005). As previously mentioned, NFAT can activate hypertrophic response gene expression. However, NFAT is inhibited by GSK3 and also negatively regulated by JNK (Dorn and Force, 2005; Schulz and Yutzey, 2004). Thus, expression of the ERRγ target, *Map3k4*, forms yet another point of regulation in the hypertrophic program since it can activate JNK (Davis, 2000). It is known that MEF2 can cooperate with NFAT to activate the expression of hypertrophic response genes (McKinsey and Olson, 2005). Although, MEF2 can be inhibited by HDAC 5 or 9, these histone deacetylases are inactivaed by PLC mediated activation of PKCs and CaMK, therefore promoting cardiac hypertrophy (McKinsey and Olson, 2005; McKinsey et al., 2002). Collectively, these results show a role other than energy metabolism for ERRα and ERRγ through their transcriptional control of genes linked to cardiac hypertrophy and cardiomyopathy.

DISCUSSION

The number of ERRα targets, and to a lesser extent that of ERRγ identified to date, have been limited. In this study, ChIP-on-chip was optimized and performed using C57BL/6J mouse hearts to provide further insight into the roles of these orphan nuclear receptor ERR isoforms through identification of novel direct target genes on a genome-wide basis. C57BL/6J mice were used this study since mice of this background were used to generate *Esrra*-null mice in our laboratory. Very few publications to date involve ChIP-on-chip using primary tissues for the genome-wide identification of transcription factor binding sites. Of these, tissues such as the liver or pancreas have been used (Odom et al., 2004; Phuc Le et al., 2005). No publication to our knowledge has shown ChIP-on-chip data obtained using murine hearts.

Target validation of the triplicate ERRα and ERRγ ChIP-on-chip experiments performed, which entails the use of standard ChIP and quantitative PCR to assess transcription factor occupancy at certain promoters, established a strong confidence of true-positive targets at a cutoff of p-value ≤ 0.01. This resulted in a total of 194 and 229 ERRα and ERRγ targets, respectively, of which 88 are in common with both receptors. All the targets validated, ranging in genes with the lowest p-values to those with p-values ≤ 0.01, were all found to be true ERRα and/or ERRγ targets, having been confirmed by standard ChIP and quantitative PCR. This finding strongly suggests that a number of ERRα and ERRγ target genes are excluded from the list of genes validated supporting the need for further validation of genes with p-values > 0.01. For example, *Rara* and *Pdk4*,

were not included in the target gene list as ERRα targets, each being associated with a p-value > 0.01, but were found to be enriched by ERRα in standard ChIP.

The significant overlap in ERRα and ERRγ target genes observed suggests functional redundancy of the receptors and possible competition for binding to response elements. Another plausible explanation is the idea that the two ERR isoforms recognize a common set of genes as heterodimeric partners. Future sequential ChIP experiments may help to address if in fact ERRα-ERRγ heterodimerization exists to regulate a subset of target promoters.

Some of the ERRα promotors obtained from the location analysis include previously identified target genes of this nuclear receptor in cardiac myocytes including *Fabp3*, *Acadm*, *Cycs*, and *Atp5b* (Huss and Kelly, 2004). *Fabp3* and *Acadm* are also targets of PPARα, an important regulator of fatty acid metabolism (Huss and Kelly, 2004). Another known ERRα target is *Gapba*, which encodes the NRF-2 subunit (Mootha et al., 2004). The NRF-2 transcription factor regulates genes involved in mitochondrial biogenesis, electron transport and oxidative phosphorylation (Huss and Kelly, 2004; Scarpulla, 2002). ERRα regulation of the *Gapba*, *Acadm*, *Cycs* and *Atp5b* genes has been shown to occur through PGC-1α activation (Huss and Kelly, 2004; Huss and Kelly, 2005; Mootha et al., 2004; Schreiber et al., 2004; Sladek et al., 1997). In fact, studies on cardiomyocytes have demonstrated that PGC-1α can induce mitochondrial proliferation, stimulate mitochondrial gene expression and increase levels of fatty acid oxidation and oxidative phosphorylation (Huss et al., 2004; Lehman et al., 2000). Although both ERRα and ERRγ are known to mediate PGC-1α regulation of cardiac energy metabolism, now more in depth knowledge of the roles of these transcription

factors in the mouse heart is possible from the novel direct target genes of these orphan receptors identified from ChIP-on-chip analyses (Huss and Kelly, 2004). A large number of the ERRα and ERRγ targets found are involved in energy metabolic processes including carbohydrate (i.e. *Clybl*, *H6pd*), lipid (i.e. *Pla2g12a*, *Sptlc2*), and protein (i.e. *Eif2b2*, *Mrp147*) metabolism as well as electron transport/oxidative phosphorylation (i.e. *Ndufs7*, *Sdhb*). Overall, these findings provide additional support of the previous notion of these ERR receptors as important regulators of energy metabolism, however, a more intriguing finding from the genome-wide location experiments, is the ERRα and ERRγ target genes associated with cardiac hypertrophy.

Cardiac hypertrophy is a compensatory mechanism to maintain cardiac energy requirements and stabilize the increased wall pressure that can result from a natural response to various cardiovascular diseases such as myocardial infarction or hypertension or through induction by means such as transverse aortic constriction (TAC) (De Acetis et al., 2005; Gao et al., 2005; Wang et al., 2004). Physiological changes associated with cardiac hypertrophy include increased cardiac pumping, enhanced expression of contractile proteins and metabolic enzymes, increased cardiomyocyte size, release and secretion of neurohumoral mediators, growth factors, and cytokines (De Acetis et al., 2005; Xu et al., 2004). Increased cardiac workload, as seen during cardiac hypertrophy, results in increased oxygen consumption and a switch from preferred fatty acid oxidation to increased reliance on glucose metabolism (Taegtmeyer et al., 2002). This cardiac hypertrophy compensatory response may evolve into decompensated hypertrophy associated with cardiac dilatation and contractile dysfunction predisposing an individual to heart failure if the hypertrophic stimulus is maintained (Chien, 1999; De Acetis et al.,

2005; Gao et al., 2005; Miyamoto et al., 2004). Decompensatory hypertrophy can also result from myocyte death and deregulated Ca^{2+} cycling (De Acetis et al., 2005).

Much of our understanding of intracellular signaling pathways implicated in cardiac hypertrophy stems from studies on induced cardiac hypertrophic response in mice, chronic pressure overload mouse models and cardiomyocyte cells in culture. Signaling pathways known to play a role in the cardiac hypertrophic program include PKCs, PI3K/Akt, MAPK, JAK/STAT, calcium-calmodulin-dependent kinases, calmodulin-dependent proteases, and calcineurin (Dorn and Force, 2005; Miyamoto et al., 2004). Although the exact molecular mechanisms involved in the transition from compensated to decompensated hypertrophy are not completely understood, many efforts have been undertaken to gain further insight into the signaling pathways and molecules involved in the hope of finding therapeutic targets for the prevention of unfavorable cardiac hypertrophy, a precursor of heart failure.

This study has implicated both ERRα and ERRγ in cardiac hypertrophy through their transcriptional control of factors associated with a number of signaling pathways involved in the cardiac hypertrophic response especially revolving around serotonin Gq-coupled 5-hydroxytryptamine (serotonin) receptor 2B activation. Activation of the Htr2b hypertrophic program leads to increased Sp1 expression and activity, activation of the PI3k/Akt and ERK pathways as well as PLC mediated DAG production and activation of calcium/calmodulin signaling (Dorn and Force, 2005; Huss and Kelly, 2005; Nebigil and Maroteaux, 2003). Together these activated pathways lead to physiological responses characteristic of cardiac hypertrophy including a greater reliance on glucose oxidation with a decrease in fatty acid oxidation, increased mitochondrial proliferation and protein

synthesis and activation of genes involved in the hypertrophic response program (Dorn and Force, 2005; Huss and Kelly, 2004; Huss and Kelly, 2005). It has previously been established that Htr2b-null mice display a dilated cardiomyopathy-like phenotype, showing left ventricular dilatation, decreased cardiomyocyte number and contractility and impaired oxidative phosphorylation and respiration (Nebigil and Maroteaux, 2003). Gq-coupled Htr2b activation of PI3K/Akt and ERK pathways which lead to the inhibition of ANT-1 and BAX expression, respectively, is absent in the Htr2b knock-out mice (Nebigil and Maroteaux, 2003). Thus, absence of Htr2b, results in overexpression of ANT-1 and BAX in turn leading to increased mitochondrial permeability followed by cytochrome c release, caspase activation and cardiomyocyte death (Nebigil and Maroteaux, 2003).

Apoptosis has only been recently discovered to occur in hypertrophic and failing hearts associated with cardiac dilatation, and thought to contribute to the switch from compensated to decompensated heart failure (Bennett, 2002; Garg et al., 2005; Nadal-Ginard et al., 2003; Razavi et al., 2005; Sharma et al., 2004; Wencker et al., 2003). Cardiomyocyte apoptosis may contribute to cardiac dysfunction since a loss of cardiomyocytes leads to reduced cardiac mass resulting in decreased cardiac pumping capacity as well as cardiac remodeling due to a redistribution of surrounding viable cardiomyocytes (Tao et al., 2005). An intriguing study found that cardiomyocyte death in failing human hearts was found to occur less frequently in women than in men resulting in a delayed onset of heart failure (Guerra et al., 1999; Isles et al., 1992). This observed phenomenon may be due in part to the higher level of estrogen in women compared to men since estrogen receptor signaling is known to activate the PI3K/Akt survival pathway, likely reducing and/or delaying cardiomyocyte death (Morissette and

Rosenzweig, 2005). Studies have demonstrated that Akt activation can prevent cardiomyocyte apoptosis (Communal and Colucci, 2005; Matsui and Rosenzweig, 2005). In addition, as previously mentionned, ERRs and ERs have similar transactivation properties. Thus, it would be interesting to investigate this observed sex-dependent onset of heart failure by evaluating whether some of the ERRα and ERRγ targets found associated with cardiac hypertrophy are also regulated by ER signaling. In any case, it is important to note that only hearts from male mice were used for the ERRα and ERRγ ChIP-on-chip studies.

Aside from the different signaling pathways involved in cardiac hypertrophy, apoptosis, being a highly ordered process, is a potential therapeutic target for the prevention of cardiac failure since inhibition of apoptosis leads to reduced cardiac dysfunction and mortality (Hayakawa et al., 2003; Kitsis and Mann, 2005; Wencker et al., 2003). ChIP-on-chip analyses resulted in the finding of a fair number of ERRα and ERRγ targets associated with apoptosis. Of particular interest, the ERRα target, *Aven*, encoding an apoptosis caspase activation inhibitor was found. This target gene favors compensated versus decompensated hypertrophy. Taken together, ChIP-on-chip reveals ERRα and ERRγ as regulators of cardiac hypertrophic signaling pathways with a number of targets favoring compensated versus decompensated hypertrophy.

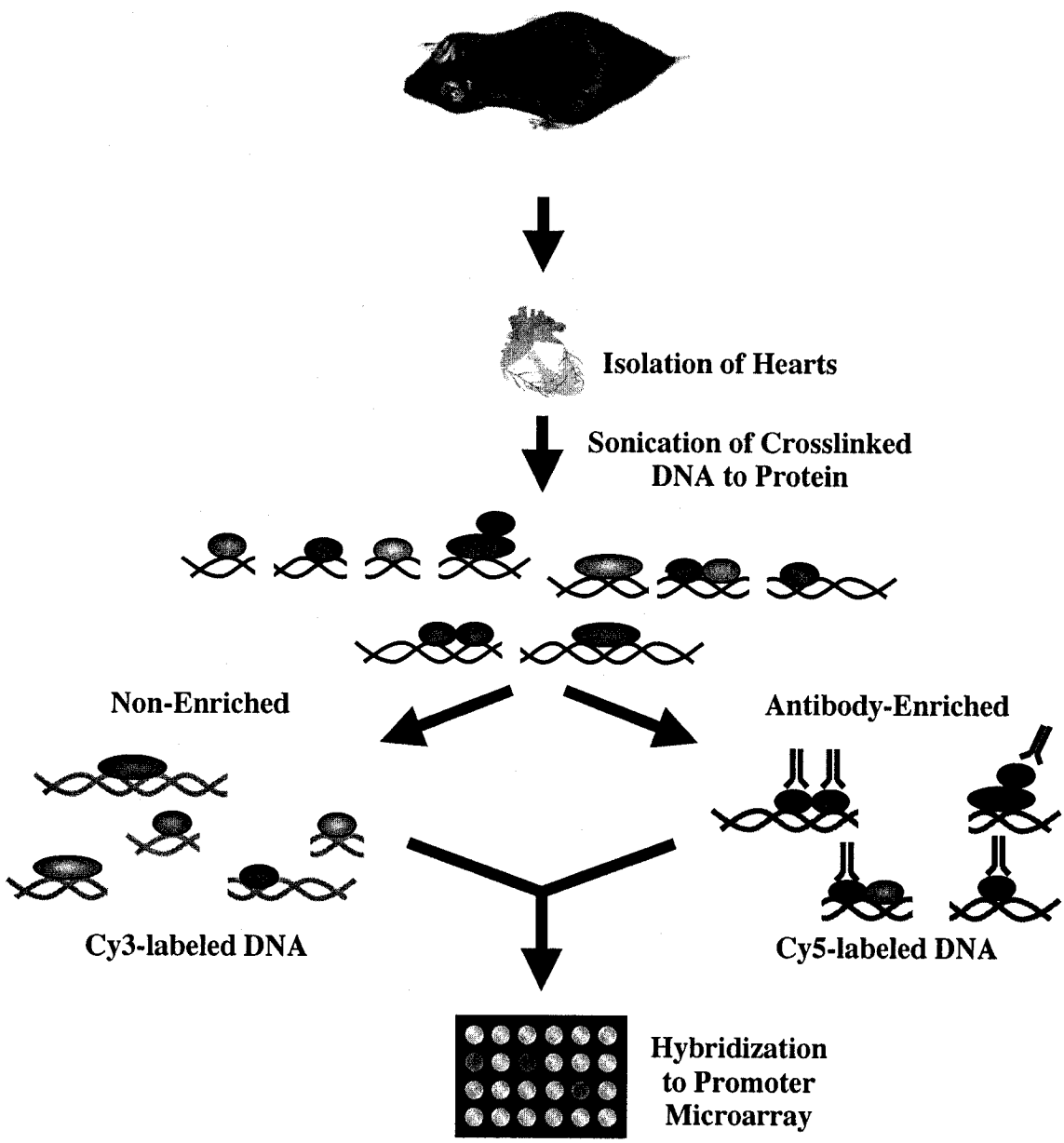
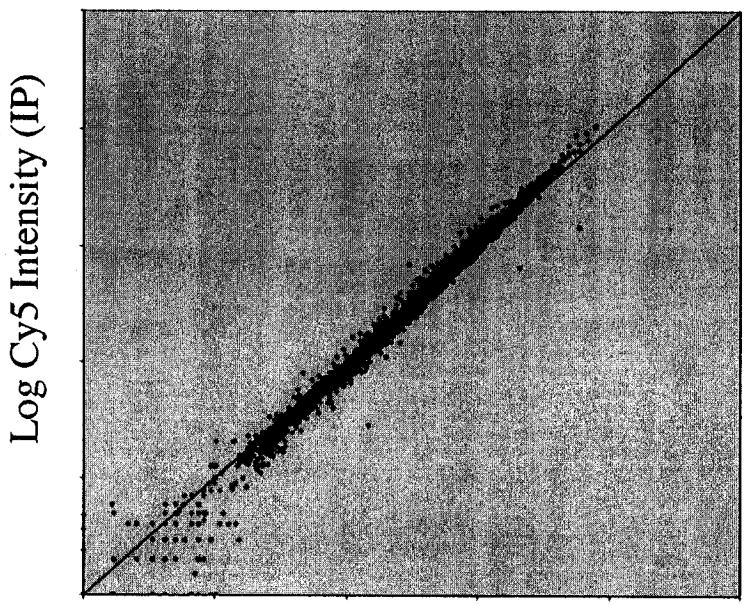

Isolation of Hearts
Sonication of Crosslinked DNA to Protein
Non-Enriched
Antibody-Enriched
Cy3-labeled DNA
Cy5-labeled DNA
Hybridization to Promoter Microarray

Figure 2: Schematic of mouse ChIP-on-chip technique. Male mouse hearts are isolated and genomic DNA is crosslinked to protein by formaldehyde treatment prior to chromatin sonication. Antibody-specific enriched and control input DNA are labeled with Cy5 and Cy3 dye, respectively, and hybridized to mouse 19K promoter microarrays. Spots (red) with a more intense signal for enriched DNA relative to control input DNA are potential direct target genes.

A


Log Cy5 Intensity (IP)
Log Cy3 Intensity (WCE)

B

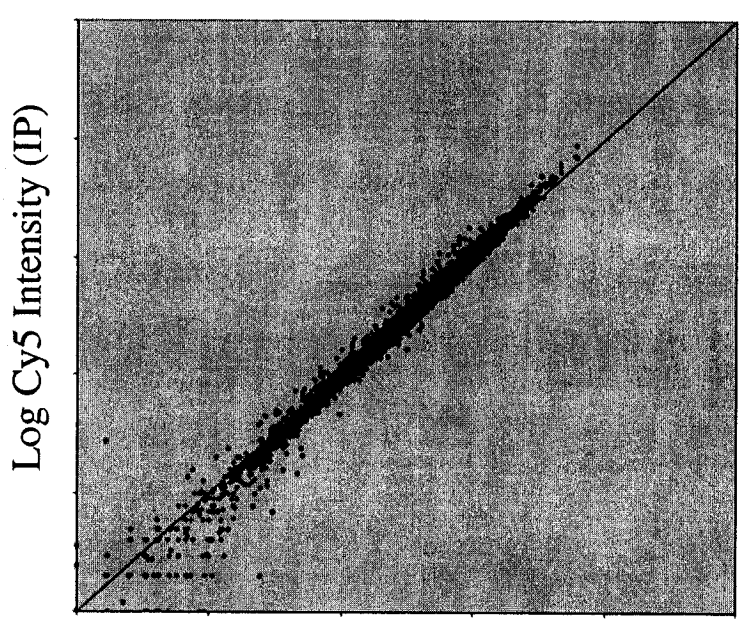

Log Cy5 Intensity (IP)
Log Cy3 Intensity (WCE)

Figure 3: Scatter plots representing triplicate ERRα (A) and ERRγ (B) location analyses for gene target identification on mouse 19K promoter microarrays using murine hearts. Intensities of Cy5-labeled ERRα- or ERRγ-specific antibody enriched samples are plotted against Cy3-labeled input chromatin (whole-cell extract, WCE). Positive transcription factor target genes are represented by spots found above the linear curves associated with greater Cy5 versus Cy3 intensities.

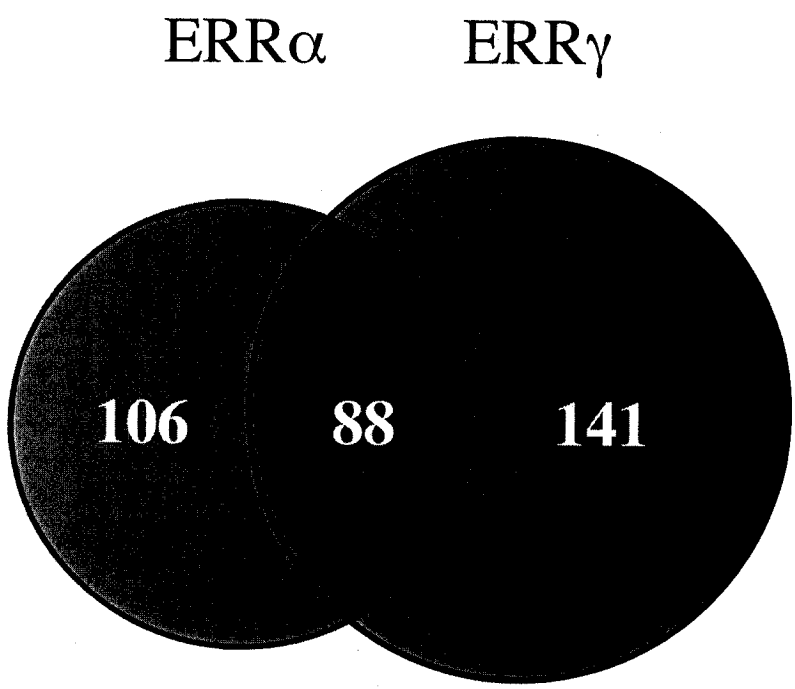
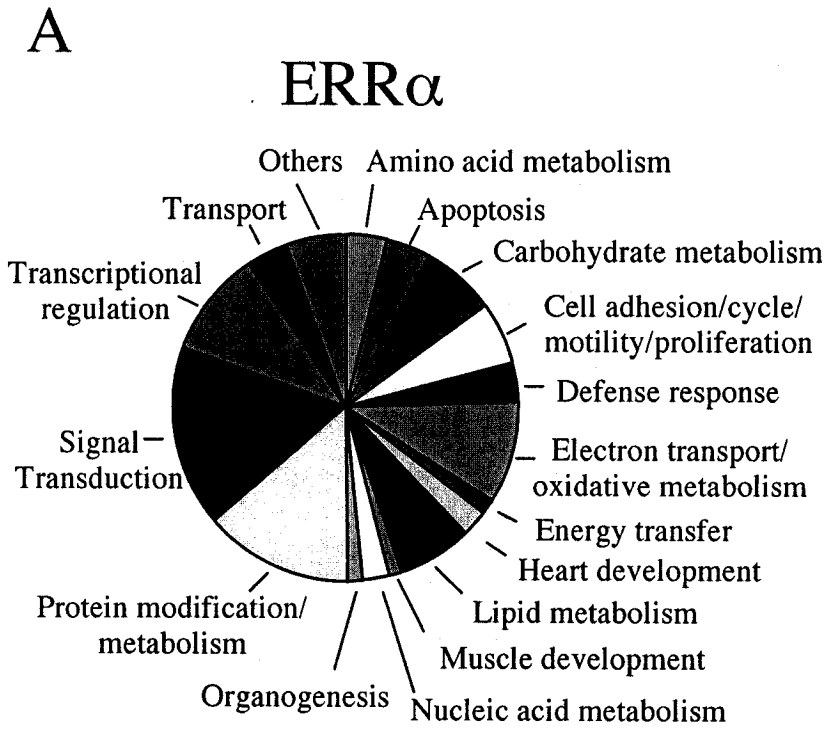
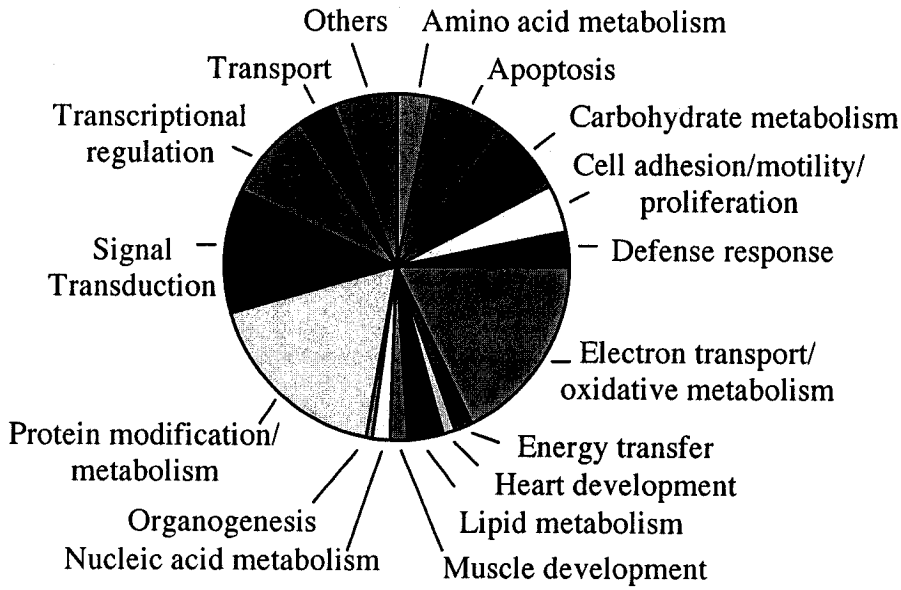

ERRα
ERRγ
106
88
141

Figure 4: Venn Diagram comparing ERRα and ERRγ targets identified from ChIP-on-chip analyses in mouse heart at a cutoff of p-value ≤ 0.01.

A


ERRα
Others
Amino acid metabolism
Transport
Apoptosis
Carbohydrate metabolism
Transcriptional regulation
Cell adhesion/cycle/ motility/proliferation
Defense response
Signal Transduction
Electron transport/ oxidative metabolism
Energy transfer
Heart development
Protein modification/ metabolism
Lipid metabolism
Muscle development
Organogenesis
Nucleic acid metabolism

B


ERRγ
Others
Amino acid metabolism
Transport
Apoptosis
Transcriptional regulation
Carbohydrate metabolism
Cell adhesion/motility/ proliferation
Signal Transduction
Defense response
Electron transport/ oxidative metabolism
Protein modification/ metabolism
Energy transfer
Heart development
Organogenesis
Lipid metabolism
Nucleic acid metabolism
Muscle development

C

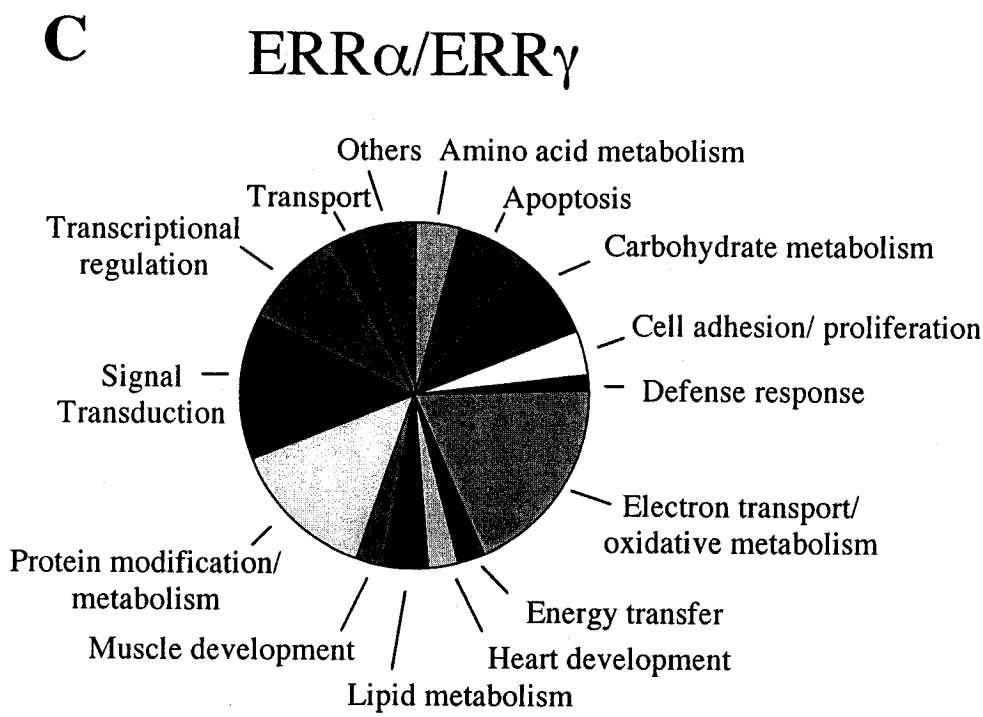

ERRα/ERRγ
Others
Amino acid metabolism
Transport
Apoptosis
Transcriptional regulation
Carbohydrate metabolism
Cell adhesion/ proliferation
Signal Transduction
Defense response
Electron transport/ oxidative metabolism
Protein modification/ metabolism
Energy transfer
Muscle development
Heart development
Lipid metabolism

Figure 5: Pie charts representing major cellular functions of target genes of ERRα (A), ERRγ (B), and those common to both ERRα and ERRγ (C) with assigned functions in mouse heart at a cutoff of p-value ≤ 0.01.

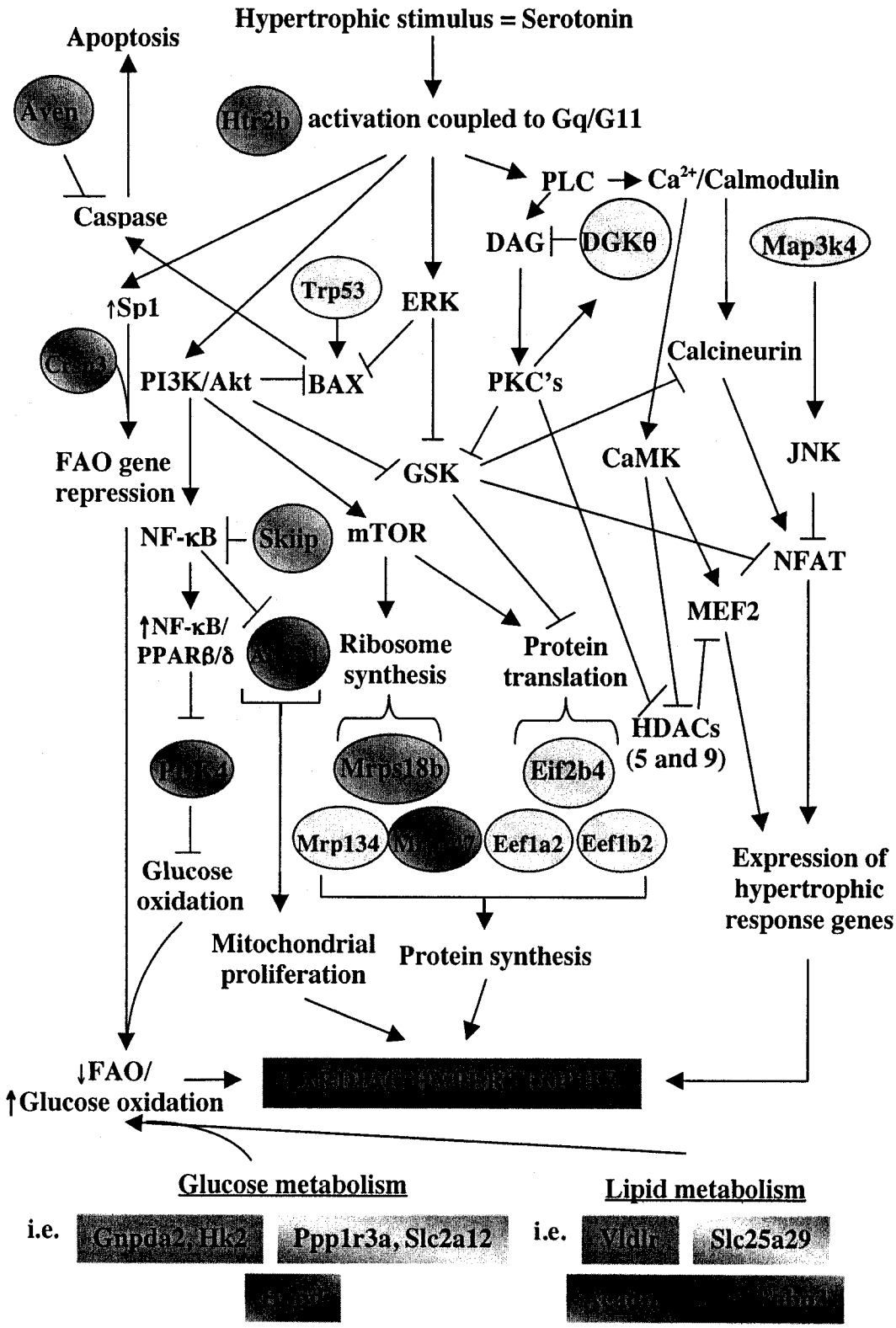

Hypertrophic stimulus = Serotonin
Apoptosis
Aven
Htr2b
activation coupled to Gq/G11
PLC
Ca2+/Calmodulin
Caspase
DAG
DGKθ
Map3k4
Trp53
ERK
↑Sp1
PI3K/Akt
BAX
PKC's
Calcineurin
FAO gene repression
GSK
CaMK
JNK
NF-κB
Skip
mTOR
NFAT
MEF2
↑NF-κB/ PPARβ/δ
Ribosome synthesis
Protein translation
HDACs (5 and 9)
Mrps18b
Eif2b4
Mrp134
Eef1a2
Eef1b2
Glucose oxidation
Mitochondrial proliferation
Protein synthesis
Expression of hypertrophic response genes
↓FAO/ ↑Glucose oxidation
Glucose metabolism
i.e.
Gnpda2, Hk2
Ppp1r3a, Slc2a12
Lipid metabolism
i.e.
Vldlr
Slc25a29

Figure 6: ERRα and ERRγ target genes associated with serotonin Gq-coupled Htr2b activated signaling pathways implicated in cardiac hypertrophy. Upon serotonin binding to Htr2b a number of protein signaling pathways are activated. Activation of the PI3K/Akt pathway and increased Sp1 expression collectively lead to a decrease in fatty acid oxidation and increased dependence on glucose oxidation, an underlying feature of cardiac hypertrophy. PI3K/Akt signaling also leads to NF-κB activation leading to ANT-1 inhibition, an important factor in regulating mitochondrial membrane permeability. Activation of the hypertrophic program also leads to PLC mediated DAG production and Ca^{2+}/Calmodulin signaling which can in turn activate PKCs and the expression of hypertrophic genes. Activated PKCs as well as ERK and PI3K/Akt pathways can lead to GSK3 inhibition, a protein kinase capable of inhibiting the hypertrophic program through repression of protein translation factors and the expression of hypertrophic response genes. The Trp53-mediated BAX apoptotic pathway is negatively regulated by PI3k/Akt and ERK inhibition of BAX. Direct target genes of ERRα, ERRγ or of both linked to cardiac hypertrophic signaling pathways are shown in pink, yellow and blue, respectively.

Table 1: Mouse primers used for ChIP quantitative PCR analysis.

GENE	PRIMER
Acadm	forward 5'-CGATCTAGCCCAGAATTTGTTGTTCCAGTG-3' reverse 5'-CTGTGCTTGGCCCGGAGAAGAAGACTGTGT-3'
Atp5b	forward 5'-CATGTTGAGTCTTGTGGGGCGTGTGGC-3' reverse 5'-GAGAAGAACCGAACTAGCTCCTCCAGG-3'
Casq2	forward 5'-CCGTCCTCTGTATGCTGGGTCAGTAGG-3' reverse 5'-GGTCGGTTCCAGGACAGCCTTGCAGCC-3'
Ckm	forward 5'-GCCCTTTAACTTGTAACCATGTGGACCTG-3' reverse 5'-GAGAACACCACCCCCCCCTGACCCATCTCG-3'
Ckmt2	forward 5'-CCACAGGCTCCTGTTACACTTCTTGGCTGT-3' reverse 5'-GTTTCTTATTAAGCGTTGCCTACATGGTTG-3'
Commd5	forward 5'-CCTCCAATCTCTGATGAAGCAACACAC-3' reverse 5'-GACGTTTTATCAAGGATGGTATGAATG-3'
Control	forward 5'-TTGGCATTGATATTGGGGGTGGGAGCAACT-3' reverse 5'-GACTTCTTACTTTGACGCTTTCCTCCATCG-3'
Cox6c	forward 5'-GGCCCGTTTCCCCTTGTATTTTGCCGC-3' reverse 5'-CAATTCTACTCCCACACCAGGCCGACC-3'
Cybrd1	forward 5'-CCACTACACTCTCATCCCTACAGGTGCTTC-3' reverse 5'-GGAGGGCGGTGGTACACACTCTTTGTCACC-3'
Cycs	forward 5'-GCTTGCCTGCACACCCTCCTCGTTTTCTAC-3' reverse 5'-AGTGGACGCCTGCTGGTGACCTTTACTCTG-3'
Cyp27a1	forward 5'-CACAATCAGCTTCAGCCTCTGGGGAAC-3' reverse 5'-GGACTCTCAAGAACTTCGCTGGCTCTC-3'
Esrra	forward 5'-GTGGCCCCGCCTTTCCCCGTGACCTTCATT-3' reverse 5'-ACCCCTGAGGACCCTCAAGTGGAGAAGCAG-3'
Fabp3	forward 5'-GGCACAAGCTCAGAGGTCAGTAAATAAAGC-3' reverse 5'-GAGAACGTGACCTTGCTTCCCTCATGCTAG-3'
Fh1	forward 5'-CGGGGTGACCTTCACGTCAGAGTGTCC-3' reverse 5'-CTGACACTCAGGCTCCCCAGCAGGAAG-3'

Table 1: Continued.

GENE	PRIMER
Fhos2	forward 5'-CTCTGTCGCAGAAGGTCAAGTTAGAAC-3' reverse 5'-GCATTGAGAATTGGTAGACTGCAAGGAGAG-3'
Gabpa	forward 5'-CACCCCGTCTTTTCTGCTTTTCGAGTC-3' reverse 5'-CTTCTGGGTTTCTTCACGAGGAGAGAG-3'
Got1	forward 5'-CTAGTATGTGCTCATCCCTTACTTTCTGC-3' reverse 5'-CAAGTGTCTGGCACAGGACTCTAGTGGTC-3'
Hk2	forward 5'-GAGCATCACGCCAGTGACTCTGATAGGTGC-3' reverse 5'-GTACCCTAAGTCTCACTCCTGCCGACC-3'
Kcnj16	forward 5'-GCTCCAAAGCTCAAGCTCTTCTTCCTCGTC-3' reverse 5'-GGCATCTTCTGAAGCAGGCAATAACCAGTC-3'
Ldb3	forward 5'-CCTTTATCTTGGGGGTCATGCAGCACC-3' reverse 5'-CAAGGTCACACAGAAAATGGACTCAGG-3'
Man1b	forward 5'-GTGCCACCTCCTTCTTCAACCAGCCTC-3' reverse 5'-GTTCCTGGGACTGACGGGTTACGGGGG-3'
Map3k4	forward 5'-GATTGGTGTGTCACTCTGATGGTCTGAGTC-3' reverse 5'-CATTTGGAGAGGTAGGGTTGTCTGGGCGAG-3'
Mdh1	forward 5'-CGCCAGAGGTCGCCGGAAGAACTACAC-3' reverse 5'-CCAGGAGCCCACACTCACCATTATTGC-3'
Mic2l1	forward 5'-GGCTTTTGGTTGTCAGCAGCAGAGAATATC-3' reverse 5'-GAACAGTTCCCGAGACTTGCGTTAGAG-3'
Mtap2	forward 5'-GCTAACACGAAGGGGAGACTGGTTATG-3' reverse 5'-CTTGGCAATGTGTCACTTCAAGGCTAG-3'
Mtch2	forward 5'-GAAAACAACCATCAAACTTTACGTCGTC-3' reverse 5'-CGGAAGGAGTAGGGAGGGAATGGTAATG-3'
Mycbp	forward 5'-GTTATCTGCAAATATCACGGGGCTTAATGA-3' reverse 5'-GCACTCCTTGTCATTTGGGTCTTTGGTGAG-3'
Myl3	forward 5'-CAAAATGGAAGGCTACTCACAGCAGATG-3' reverse 5'-CAGTCACTGTCTCCAAAGTCAAGGTCAC-3'

Table 1: Continued.

GENE	PRIMER
Ndufa9	forward 5'-GCCTCTGCATTTCTATTTAACTCCCCC-3' reverse 5'-GAGTTAACCAAGGACTGACTCAAGGGCATG-3'
Ndufb5	forward 5'-CTTGGGATTTACTAGGGAACAAGCTGCC-3' reverse 5'-GCCTGTTCCTGTTTGCTTCACTTGCCTC-3'
Oxt	forward 5'-CAAGTCTCTCTAGCCTCTTGTAGCCTA-3' reverse 5'-GTCACAGAAGGTCTCTTGGGTCAAATTG-3'
Pdk4	forward 5'-GGAAACAGTTTCTGGCTAGGAATGCGTGA-3' reverse 5'-CTGCAAGGGGCAAAGGGTGAGAGGGAGGAGG-3'
Phactr1	forward 5'-CAGGGTTGATCAGGGACTGCCAAAAGG-3' reverse 5'-GAACGCAGCATCATACATAGCAGGGGC-3'
Pi15	forward 5'-CTATGTCACTCGGTATGCAGAATGTCTG-3' reverse 5'-GAATACTTTGCCCAAATGCTTGAACTGC-3'
Plod3	forward 5'-GCTTGAAGTCATTACGTGATGTTCCCTTGG-3' reverse 5'-CGCTCCCCTAAAGACCACCCACGCCAG-3'
Plscr2	forward 5'-GCTGAGTCGCTGCTGGTGCTAGGATTCTAG-3' reverse 5'-GTCACCTTCTTGGTCACCTTTCCTAACTTC-3'
Polr3d	forward 5'-CGGATAAATACTATGGTCAGGTCACGC-3' reverse 5'-GCCGAGTATCACAACCTCACCTCCGAC-3'
Rara	forward 5'-GAGAGGTGTTTGGGAAAGAAGGATGTTC-3' reverse 5'-CTGTCAAGTCTCTGTCTCTCCTAATCAC-3'
Sdhb	forward 5'-CTTCCTGTACATTGGCTCGGAGAAACC-3' reverse 5'-CTTCAAGGAGACCCCGACCGTCGCCGC-3'
Skiip	forward 5'-GCAGAGTTGGATAATAGTATTAGCGTCCC-3' reverse 5'-GACTTGTGACAGCCAGTCTGTTGAAAG-3'
Slc25a4	forward 5'-CAAACGAGCGGCTCCTTGCAGGCTGTG-3' reverse 5'-TGCAGCAGCAGTTTGACCCTCTCGATC-3'
Slc25a29	forward 5'-GTTCTCGAACTTTCCCAGCAAGCTGAGTC-3' reverse 5'-GATCTGCTTCAATCCTCGGACCCCTCACG-3'

Table 1: Continued.

GENE	PRIMER
Slco1b2	forward 5'-CTGCATGGTACTGGTACAATGACAGACAGG-3'
	reverse 5'-GCATTCTTCTGCATGATAACTGCCACG-3'
Sptlc2	forward 5'-GCAGAGGAACATAGAGGTCACTCAGCTAG-3'
	reverse 5'-CAAATCAGTAGCAAAAGAAATTGATCTAC-3'
Stk2	forward 5'-GCCACAAACTTTGCCTTTTATTGTTCC-3'
	reverse 5'-CTCTTCGGGGTTCAGGTGTCTCTTCAC-3'
Tcap	forward 5'-CATGTGCCTGGTCCGAGGTGGTGTTTG-3'
	reverse 5'-CAAGACTCACTGGTGGTGATGGGGCTC-3'
Timm8b	forward 5'-GCAGAGAACGCCCAGCTTTAAGAGAACC-3'
	reverse 5'-CTCCTGCCTTCCGGTTCACGCTTCAGG-3'
Vldlr	forward 5'-GAGCTGTCAGAGCCGAGGTTACTGGGAGCC-3'
	reverse 5'-CTCCAAAGCCCACATCCCCTAGCCCTTGTC-3'

Table 2: Cellular functional classification of target genes occupied by ERRα, ERRγ or both in mouse heart at a cutoff of p-value ≤ 0.01.

GENE	DESCRIPTION	ERRα	ERRγ
Amino Acid Metabolism			
As3mt	Arsenic (+3 oxidation state) methyltransferase		x
Bhmt	Betaine-homocysteine methyltransferase		x
Cbs	Cystathionine beta-synthase	x	
Got1	Glutamate oxaloacetate transaminase 1, soluble	x	x
Got2	Glutamate oxaloacetate transaminase 2, mitochondrial		x
Gpt1	Glutamic pyruvic transaminase 1, soluble		x
Oplah	5-Oxoprolinase (ATP-hydrolysing)	x	x
Pah	Phenylalanine hydroxylase	x	
Thnsl1	Threonine synthase-like 1 (bacterial)	x	
Apoptosis			
Als2cr2	Amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 2 (human)		x
Aven	Apoptosis, caspase activation inhibitor	x	
Bdnf	Brain derived neurotrophic factor	x	x
Cd59a	CD59a antigen	x	x
Dad1	Defender against cell death 1		x
Eef1a2	Eukaryotic translation elongation factor 1 alpha 2		x
Endog	Endonuclease G		x
Ets1	E26 avian leukemia oncogene 1, 5' domain		x
Fgl2	Fibrinogen-like protein 2	x	x
Mtch2	Mitochondrial carrier homolog 2 (C. elegans)	x	x
Pdcd7	Programmed cell death protein 7	x	
Pdcl3	Phosducin-like 3		x
Rabep1	Rabaptin, RAB GTPase binding effector protein 1		x
Stk2	Serine-threonine kinase 2	x	x
Trp53	Tumor protein p53		x
Carbohydrate Metabolism			
1810014F10Rik	RIKEN cDNA 1810014F10 gene	x	x
2310032D16Rik	RIKEN cDNA 2310032D16 gene		x
Chst3	Carbohydrate (chondroitin 6/keratan) sulfotransferase 3	x	
Clybl	Citrate lyase beta like	x	x
Fuk	Fucokinase	x	
Gnpda2	Glucoseamine-6-phosphate deaminase 2	x	
H6pd	Hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase)	x	x
Hdhd3	Haloacid dehalogenase-like hydrolase domain containing 3		x
Hk2	Hexokinase 2	x	
Ldh2	Lactate dehydrogenase 2, B chain	x	x
Man1b	Mannosidase, alpha, class 1A, member 2	x	
Me3	Malic enzyme 3, NADP(+)-dependent, mitochondrial		x

Table 2: Continued

GENE	DESCRIPTION	ERRα	ERRγ
Muc15	Mucin 15	x	x
Neu3	Neuraminidase 3		x
Ppp1r3a	Protein phosphatase 1, regulatory (inhibitor) subunit 3A		x
Slc2a12	Solute carrier family 2 (facilitated glucose transporter), member 12 (GLUT-12)		x
Stxbp3	Syntaxin binding protein 3	x	x
Cell Adhesion			
Cd6	CD6 antigen	x	
Cdh26	Cadherin-like 26	x	x
Nrxn3	Neurexin III	x	
Pcdha6	Protocadherin alpha 6	x	
Rs1h	Retinoschisis 1 homolog (human)	x	x
Ssx2ip	Synovial sarcoma, X breakpoint 2 interacting protein		x
Tnr	Tenascin R	x	
Cell Cycle			
Spin	Spindlin	x	
Cell Motility			
Dnali1	Dynein, axonemal, light intermediate polypeptide 1	x	
Ferd3l	Fer3-like (Drosophila)		x
Pxn	Paxillin		x
Cell Proliferation			
Commd5	COMM domain containing 5	x	x
Cspg4	Chondroitin sulfate proteoglycan 4		x
Nmyc1	Neuroblastoma myc-related oncogene 1		x
Chromosome Biogenesis			
Cenpa	Centromere autoantigen A	x	x
Cytoskeleton			
FHOS2	Formin-family protein FHOS2		x
Ipp	IAP promoted placental gene	x	
Klhl5	Kelch-like 5 (Drosophila)		x
Krtap4-7	Keratin associated protein 4-7	x	
Mtap2	Microtubule-associated protein 2	x	
Ppl	Periplakin		x
Defense Response			
Abhd8	Abhydrolase domain containing 8		x
Ccr3	Chemokine (C-C motif) receptor 3		x
Clecsf12	C-type (calcium dependent, carbohydrate recognition domain) lectin, superfamily member 12	x	
Defb41	Defensin beta 41	x	
Hspa9a	Heat shock protein 9A	x	x
Hus1	Hus1 homolog (S. pombe)	x	
Krt2-8	Keratin complex 2, basic, gene 8		x
Map3k4	Mitogen activated protein kinase kinase kinase 4		x

Table 2: Continued

GENE	DESCRIPTION	ERRα	ERRγ
Ppp1r10	Protein phosphatase 1, regulatory subunit 10	x	
Reg3a	Regenerating islet-derived 3 alpha	x	
Ugt2a3	UDP glucuronosyltransferase 2 family, polypeptide A3		x
DNA Repair			
Smc6l1	SMC6 structural maintenance of chromosomes 6-like 1 (yeast)	x	
Electron Transport/Oxidative Metabolism			
1110020P15Rik	RIKEN cDNA 1110020P15 gene		x
Atp5b	ATP synthase, H+ transporting, mitochondrial F1 complex, beta subunit	x	x
Atp5c1	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1		x
Atp5f1	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit b, isoform 1		x
Atp5g1	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c, (subunit 9), isoform 1		x
Atp5g3	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c, (subunit 9), isoform 3	x	x
BC019806	cDNA sequence BC019806	x	x
Coq7	Demethyl-Q-7	x	x
Cox6c	Cytochrome c oxidase, subunit VIc	x	x
Cox8a	Cytochrome c oxidase, subunit VIIIa		x
Cox8b	Cytochrome c oxidase, subunit VIIIb		x
Cybrd1	Cytochrome b reductase 1	x	
Cycs	Cytochrome c, somatic	x	x
Cyp27a1	Cytochrome P450, family 27, subfamily a, polypeptide 1		x
Etfb	Electron transferring flavoprotein, beta polypeptide		x
Etfdh	Electron transferring flavoprotein, dehydrogenase		x
Fh1	Fumarate hydratase 1	x	x
Mdh1	Malate dehydrogenase 1, NAD (soluble)		x
Ndufa4	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4		x
Ndufa8	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 8		x
Ndufa9	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 9	x	x
Ndufb2	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2		x
Ndufb4	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 4	x	x
Ndufb5	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5	x	x
Ndufs1	NADH dehydrogenase (ubiquinone) Fe-S protein 1		x

Table 2: Continued

GENE	DESCRIPTION	ERRα	ERRγ
Ndufs2	NADH dehydrogenase (ubiquinone) Fe-S protein 2		x
Ndufs7	NADH dehydrogenase (ubiquinone) Fe-S protein 7	x	x
Ndufv1	NADH dehydrogenase (ubiquinone) flavoprotein 1		x
Sdha	Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	x	x
Sdhb	Succinate dehydrogenase complex, subunit B, iron sulfur (Ip)	x	x
Sdhd	Succinate dehydrogenase complex, subunit D, integral membrane		x
Txn2	Thioredoxin 2		x
Txnl2	Thioredoxin-like 2	x	x
Energy Transfer			
Ckm	Creatine kinase, muscle		x
Ckmt2	Creatine kinase, mitochondrial 2	x	x
Slc25a4	Solute carrier family 25 (mitochondrial carrier, adenine nucleotide translocator), member 4	x	x
Heart Development			
Casq2	Calsequestrin 2	x	x
Htr2b	5-hydroxytryptamine (serotonin) receptor 2B	x	
Oxt	Oxytocin	x	x
Phc1	Polyhomeotic-like 1 (Drosophila)	x	
Immune Response			
Cd83	CD83 antigen	x	
Ctss	Cathepsin S	x	
Igj	Immunoglobulin joining chain		x
Lipid Metabolism			
Acadm	Acetyl-Coenzyme A dehydrogenase, medium chain	x	x
Ankrd9	Ankyrin repeat domain 9	x	
Ankrd23	Ankyrin repeat domain 23	x	
Cerk	Ceramide kinase	x	x
Fabp3	Fatty acid binding protein 3, muscle and heart		x
Mir16	Membrane interacting protein of RGS16	x	x
Mttp	Microsomal triglyceride transfer protein	x	
Osbpl3	Oxysterol binding protein-like 3	x	
Pla2g12a	Phospholipase A2, group XIIA	x	
Plscr2	Phospholipid scramblase 2	x	
Pte2a	Peroxisomal acyl-CoA thioesterase 2A	x	
Slc25a29	Solute carrier family 25 (mitochondrial carrier, palmitoylcarnitine transporter), member 29		x
Sptlc2	Serine palmitoyltransferase, long chain base subunit 2		x
Vldlr	Very low density lipoprotein receptor	x	
Muscle Development			
Eno1	Enolase 1, alpha non-neuron	x	x

Table 2: Continued

GENE	DESCRIPTION	ERRα	ERRγ
Myl3	Myosin, light polypeptide 3	x	x
Tcap	Titin-cap		x
Nucleic Acid Metabolism			
Exosc2	Exosome component 2	x	x
Polr3d	Polymerase (RNA) III (DNA directed) polypeptide D		x
Rbm27	RNA binding motif protein 27	x	
Rbms2	RNA binding motif, single stranded interacting protein 2	x	x
Sart3	Squamous cell carcinoma antigen recognized by T-cells 3	x	
Organogenesis			
Rara	Retinoic acid receptor, alpha		x
Tuft1	Tuftelin 1	x	
Vegfc	Vascular endothelial growth factor C	x	
Oxidoreductase			
BC034099	cDNA sequence BC034099		x
Zadh1	Zinc binding alcohol dehydrogenase, domain containing 1	x	
Polyamine Biosynthesis			
Oaz1	Ornithine decarboxylase antienzyme		x
Protein Modification/Metabolism			
5330414D10Rik	RIKEN cDNA 5330414D10 gene		x
Adam3	A disintegrin and metalloprotease domain 3 (cyritestin)	x	
Ahsa1	Activator of heat shock 90kDa protein ATPase homolog 1	x	x
Ap1g2	Adaptor protein complex AP-1, gamma 2 subunit		x
Apoa1bp	Apolipoprotein A-I binding protein	x	x
Arf1	ADP-ribosylation factor 1		x
Arf4	ADP-ribosylation factor 4	x	x
Arih2	Ariadne homolog 2 (Drosophila)		x
Art4	ADP-ribosyltransferase 4		x
D10Ertd322e	NA segment, Chr 10, ERATO Doi 322, expressed		x
Eef1b2	Eukaryotic translation elongation factor 1 beta 2		x
Eif2b4	Eukaryotic translation initiation factor 2B, subunit 4 delta		x
Gtl6	Gene Trap locus 6	x	
Grwd1	Glutamate-rich WD repeat containing 1	x	
Gtpbp4	GTP binding protein 4	x	x
Hbs1l	Hbs1-like (S. cerevisiae)	x	
Kin	Kin of IRRE like 2 (Drosophila)		x
Lman2	Lectin, mannose-binding 2	x	x
Lmln	Leishmanolysin-like (metallopeptidase M8 family)	x	
Mmp20	Matrix metalloproteinase 20 (enamelysin)	x	x
Mrpl34	Mitochondrial ribosomal protein L34		x
Mrp147	Mitochondrial ribosomal protein L47	x	x
Mrps18b	Mitochondrial ribosomal protein S18B	x	
Nifun	NifU-like-N-terminal domain containing	x	
Nktr	Natural killer tumor recognition sequence		x

Table 2: Continued

GENE	DESCRIPTION	ERRα	ERRγ
Nmt1	N-myristoyltransferase 1		x
Ormdl1	ORM1-Like 1 (S. cerevisiae)		x
Pdk4	Pyruvate dehydrogenase kinase, isoenzyme 4		x
Phactr1	Phosphatase and actin regulator 1	x	
Pi15	Protease inhibitor 15	x	
Plod3	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3		x
Ppia	Peptidylprolyl isomerase A		x
Ppif	Peptidylprolyl isomerase F (cyclophilin F)		x
Ptpn18	Protein tyrosine phosphate, non-receptor type 18		x
Qrsl1	Gutaminyl-tRNA synthase (glutamine-hydrolyzing)-like 1		x
Rab21	RAB21, member RAS oncogene family	x	x
Rpl3l	Ribosomal protein L3-like	x	x
Rnf187	Ring finger protein 187	x	
Sec61g	SEC61, gamma subunit	x	
Timm8b	Translocator of inner mitochondrial membrane 8 homolog		x
Timm17a	Translocator of inner mitochondrial membrane 17a		x
Trim39	Tripartite motif protein 39	x	
Usp52	Ubiquitin specific protease 52		x
Zcsl2	Zinc finger, CSL domain containing 2	x	x
Signal Transduction			
2610312B22Rik	RIKEN cDNA 2610312B22 gene	x	
5730466P16Rik	RIKEN cDNA 5730466P16 gene	x	x
9830160G03Rik	RIKEN cDNA 9830160G03 gene		x
Centd2	Centaurin, delta 2		x
Crtam	Cytotoxic and regulatory T cell molecule	x	x
Dgkθ	Diacylglycerol kinase, theta		x
Gnb2	Guanine nucleotide binding protein, beta 2	x	
Gsbs	G substrate	x	x
Il15ra	Interleukin 15 receptor, alpha chain	x	
Ldb3	LIM domain binding 3		x
Mapk8ip2	Mitogen activated protein kinase 8 interacting protein 2	x	x
Nadk	Nad kinase	x	x
Olfr131	Olfactory receptor 131	x	
Olfr149	Olfactory receptor 149	x	
Olfr460	Olfactory receptor 460	x	
Olfr558	Olfactory receptor 558	x	
Olfr653	Olfactory receptor 653	x	
Olfr667	Olfactory receptor 667	x	
Olfr692	Olfactory receptor 692	x	
Olfr802	Olfactory receptor 802	x	x
Olfr871	Olfactory receptor 871	x	x
Olfr893	Olfactory receptor 893	x	
Olfr895	Olfactory receptor 895	x	x

Table 2: Continued

GENE	DESCRIPTION	ERRα	ERRγ
Olfr1019	Olfactory receptor 1019		x
Olfr1136	Olfactory receptor 1136		x
Olfr1342	Olfactory receptor 1342	x	
Olfr1431	Olfactory receptor 1431	x	
Pde6g	Phosphodiesterase gG, cGMP-specific, rod, gamma	x	
Plpi	Prolactin like protein I	x	x
Rala	V-ral simian leukemia viral oncogene homolog A (ras related)	x	x
Rit1	Ras-like without CAAX 1	x	
Snx3	Sorting nexin 3	x	
Snx17	Sorting nexin 17		x
Tbc1d4	TBC1 domain family, member 4		x
Tm4sf3	Transmembrane 4 superfamily member 3		x
Vlrc7	Vomeronasal 1 receptor, C7		x
Vlre1	Vomeronasal 1 receptor, E1	x	
Spermatogenesis			
Acrbp	Proacrosin binding protein		x
Spata11	Spermatogenesis associated 11	x	x
Theg	Testicular haploid expressed gene		x
Transcriptional Regulation			
AI591476	Expressed sequence AI591476	x	x
BC034204	cDNA sequence BC034204	x	
Crsp3	Cofactor required for Sp1 transcriptional activation, subunit 3	x	x
Esrra	Estrogen related receptor, alpha	x	x
Gabpa	GA repeat binding protein, alpha	x	x
Gsc	Goosecoid	x	
Helic1	Helicase, ATP binding 1		x
Mycbp	C-myc binding protein	x	x
Phf5a	PHD finger protein 5A	x	x
Phtf2	Putative homeodomain transcription factor 2		x
Pias3	Protein inhibitor of activated STAT3	x	
Rai17	Retinoic acid induced 17	x	
Recc1	Replication factor C1		x
Rpo1-4	RNA polymerase 1-4		x
Skiip	SKI interacting protein	x	
Snip1	Smad nuclear interacting protein 1	x	
Sp2	Sp2 transcription factor		x
Spz1	Spermatogenic Zip 1	x	
Tead3	TEA domain family member 3		x
Trrap	Transformation/transcription domain-associated protein		x
V2r16	Vomeronasal 2, receptor, 16	x	
Zfp93	Zinc finger protein 93		x

Table 2: Continued

GENE	DESCRIPTION	ERRα	ERRγ
Zfp101	Zinc finger protein 101	x	x
Zfp422	Zinc finger protein 422	x	
Transport			
Abcc6	ATP-binding cassette, sub-family C (CFTR/MRP), member 6	x	
Atp9a	ATPase, class II, type 9A		x
Atp10d	ATPase, class V, type 10D		x
Clca6	Chloride channel calcium activated 6		x
Hbb-y	Hemoglobin Y, beta-like embryonic chain	x	
Kcnj16	Potassium inwardly-rectifying channel, subfamily J, member 16	x	
Slc35E2	Solute carrier family 35, member E2		x
Slc38a3	Solute carrier family 38, member 3		x
Slc41a3	Solute carrier family 41, member 3	x	x
Slco1b2	Solute carrier organic anion transporter family, member 1	x	
Sv2b	Synaptic vesicle glycoprotein 2b	x	x
Tumor Supressor			
Csmd1	CUB and Sushi multiple domains 1		x

Cellular functional classifications were based on GO annotation (http://fatigo.org/) and NCBI gene descriptions. Hypothetical genes and genes without an assigned function are not shown. ERRα and ERRγ columns marked with « x » indicates ERRα or ERRγ targets, respectively. Incidences where individual loci could be assigned to two distinct genes, both genes were included in the functional analysis.

Table 3: ERRα and/or ERRγ target genes without an assigned function.

GENE	ERRα	ERRγ
0610009O20Rik	x	x
0610011N22Rik	x	x
0610039A15Rik		x
0710005M24Rik	x	
1010001D01Rik	x	x
1110019N10Rik		x
1700010A17Rik		x
1700020B09Rik	x	x
1700040L02Rik		x
1810031K02Rik		x
1810049H19Rik	x	
2210020M01Rik		x
2210418O10Rik		x
2310042D19Rik		x
2610044O15Rik		x
2610312E17Rik		x
2610510L01Rik		x
2700038I16Rik		x
2810422B04Rik		x
2810485I05Rik		x
3110002L15Rik		x
3110048E14Rik		x
3830408P04Rik		x
3830422K02Rik	x	
4932435O22Rik	x	x
4933409D10Rik	x	x
5133401N09Rik		x
5730521E12Rik	x	
6330578E17Rik		x
9830130M13	x	
A030001H23Rik	x	
A130022J15Rik	x	x
A730055L17Rik	x	x
A930025J12Rik		x
AB124611		x
AI413782	x	x
AI428936	x	
AI839550		x
AU021034	x	
AV249152		x
AV312086		x
BC002059	x	x
BC004004	x	

Table 3: Continued.

GENE	ERRα	ERRγ
BC018101	x	
BC021790		x
BC028949	x	
BC034902	x	
BC051227	x	x
BC055791	x	x
BC070434	x	
BC072647	x	
D15Wsu169e		x
D5Ertd593e	x	
D630004N19Rik	x	x
G630055P03Rik	x	x
Gm631	x	x
Gsdm		x
LOC432637		x
MGC6357		x
MGC67181	x	
Mic2l1		x
Pgbpll	x	
Rcl	x	
Rtn4ip1		x
Sacm1l		x
Schip1	x	
Sepm	x	
Unc50		x
Unc93b1	x	
Znhit1		x

Table 4: Validation of ERRα ChIP-on-chip target genes at a cutoff of p-value ≤ 0.01.

Gene	Relative Enrichment	p-value
Atp5b	35.5	1.57E-7
Got1	27.9	6.06E-6
Esrra	58.9	8.23E-6
Stk2	3.6	5.79E-5
Ckmt2	5.7	1.06E-4
Skiip	4.2	2.67E-4
Commd5	11.2	3.56E-4
Sdhb	15.1	5.74E-4
Oxt	3.2	7.16E-4
Phactr1	2.5	1.40E-3
Acadm	20.7	1.48E-3
Mtch2	59.7	2.34E-3
Man1b	2.4	2.44E-3
Kcnj16	2.3	2.69E-3
Gabpa	16.7	2.83E-3
Pi15	2.0	3.13E-3
Ndufb5	8.3	3.34E-3
Cycs	5.6	4.20E-3
Fh1	12.5	4.42E-3
Slc25a4	8.5	4.53E-3
Casq2	4.0	5.55E-3
Mycbp	21.3	5.58E-3
Cybrd1	2.1	6.31E-3
Ndufa9	6.7	6.65E-3
Cox6c	22.8	6.91E-3
Myl3	4.9	7.80E-3
Slcolb2	2.3	8.03E-3
Mtap2	2.4	8.79E-3
Plscr2	9.4	9.44E-3
Vldlr	2.4	9.70E-3
Hk2	3.0	9.74E-3

Table 5: Validation of ERRγ ChIP-on-chip target genes at a cutoff of p-value ≤ 0.01.

Gene	Relative Enrichment	p-value
Atp5b	45.9	5.20E-9
Ckmt2	7.3	3.09E-8
Esrra	77.7	1.12E-7
Got1	47.8	3.55E-6
Slc25a4	13.7	3.64E-6
Sdhb	15.0	9.88E-6
Tcap	2.8	5.07E-5
Pdk4	20.3	6.47E-5
Ndufa9	10.0	1.20E-4
Ndufb5	14.6	1.60E-4
Map3k4	13.0	2.75E-4
Mtch2	64.4	3.28E-4
Mdh1	15.1	3.63E-4
Gabpa	23.4	3.72E-4
Mycbp	32.2	6.17E-4
Ldb3	8.5	7.55E-4
Fabp3	8.1	7.66E-4
Cox6c	38.3	7.77E-4
Fh1	17.8	1.00E-3
Commd5	16.1	1.47E-3
Timm8b	14.5	1.50E-3
Acadm	30.9	1.82E-3
Plod3	3.2	1.95E-3
Oxt	3.7	2.00E-3
Myl3	6.9	2.01E-3
Slc25a29	12.6	2.25E-3
Stk2	3.5	2.68E-3
Sptlc2	7.8	3.82E-3
Cycs	7.6	5.55E-3
Fhos2	4.3	5.56E-3
Casq2	5.7	6.00E-3
Cyp27a1	5.7	7.04E-3
Rara	14.6	7.04E-3
Ckm	3.5	8.13E-3
Polr3d	3.2	9.39E-3
Mic2l1	5.2	9.60E-3

Table 6: ERRα and/or ERRγ target genes linked with cardiac hypertrophy, cardiomyopathy or cardiovascular disease through direct evidence or by association with known factors involved.

GENE	LINK/REFERENCE
Aven	Indirect; inhibitor of caspase (Chau et al., 2000; Nebigil and Maroteaux, 2003)
Bhmt	Direct (Weisberg et al., 2003)
Casq2	Direct (Lahat et al., 2001a; Lahat et al., 2001b; Postma et al., 2002)
Cbs	Direct (Kelly et al., 2003)
Ckm	Direct (Nahrendorf et al., 2005)
Ckmt2	Direct (Fountoulakis et al., 2005; Nahrendorf et al., 2005)
Crsp3	Indirect; Sp1 cofactor (Huss and Kelly, 2005; Sack et al., 1997; Taatjes et al., 2002)
Dgkθ	Direct (van Baal et al., 2005)
Eif2b4	Direct (Dorn and Force, 2005)
Fabp3	Direct (Binas et al., 2003; Morioka et al., 2005; Setsuta et al., 2002)
Htr2b	Direct (Nebigil and Maroteaux, 2003)
Ldb3	Direct (Arimura et al., 2004; Morita et al., 2005; Vatta et al., 2003)
Map3k4	Indirect; activator of JNK (Davis, 2000; Frey and Olson, 2003; Wang et al., 1998)
Mdh1	Direct (Lo et al., 2005)
Myl3	Direct (Morita et al., 2005)
Ndufs2	Direct (Goffart et al., 2004)
Oxt	Indirect; activated by RARα (Paquin et al., 2002)
Pah	Direct (Levy et al., 2001)
Pdk4	Direct (Planavila et al., 2005)
Phc1	Direct (Koga et al., 2002)
Pias3	Indirect; inhibitor of STAT3 and NF-κB (Chung et al., 1997; Frey and Olson, 2003; Jang et al., 2004)
Rara	Direct (Colbert et al., 1997; Kastner et al., 1997)
Skiip	Indirect; inhibitor of NF-kB (Planavila et al., 2005; Zhou et al., 2000)
Slc25a4	Direct (Fountoulakis et al., 2005; Graham et al., 1997)
Tcap	Direct (Morita et al., 2005)
Trp53	Direct (Cesselli et al., 2001; Li et al., 2005)

CONCLUSION

ChIP-on-chip was successfully applied for the identification of ERRα and ERRγ direct target genes on a genome-wide basis in the mouse heart. Using a cutoff of p-value ≤ 0.01, 194 and 229 targets of ERRα and ERRγ, respectively, were identified. Over 15% of the ERR targets were validated and all were found to be true ERRα and/or ERRγ targets. This fact along with the finding of ERRα targets with p-values > 0.01 such as *Pdk4* and *Rara* demonstrates the need for further validation of targets with p-values > 0.01, providing a more complete list of ERRα and ERRγ cardiac target genes. Of the total number of ERRα and ERRγ targets obtained, about 80% were known genes with which GO functions and NCBI gene descriptions could be assigned. ERRα was found to have more targets associated with lipid metabolism, heart development, signal transduction and transcriptional regulation compared to ERRγ. On the other hand, despite the greater number of targets identified for ERRγ, this nuclear receptor was found to regulate more genes implicated in apoptosis, protein modification/metabolism, and electron transport/oxidative phosphorylation relative to ERRα. Both ERRα and ERRγ were found to occupy a similar number of genes associated with processes such as carbohydrate metabolism, transport, and amino acid metabolism. Interestingly, approximately 40% of the ERRα and ERRγ target genes were in common, with the greatest overlap of genes involved in electron transport/oxidative phosphorylation. Thus, investigation of the possibility of gene regulation through ERRα/ERRγ heterodimerization needs to be explored. Aside from the idenfication of novel ERRα and ERRγ regulated genes in cardiac energy metabolism, the genome-wide location experiments unexpectedly revealed

evidence for ERR regulation of genes associated with cardiac hypertrophy. ERRα and ERRγ were found to control a number of genes involved in serotonin Gq-coupled Htr2b receptor signaling including *Htr2b*, *Crsp3*, *Pdk4*, *ANT-1*, *Dgkθ*, and *Map3k4* along with a number of genes needed for protein synthesis as well as glucose and lipid metabolism. In summary, direct target genes identified from the mouse heart ERRα and ERRγ ChIP-on-chip experiments allows us to expand our understanding of the roles of these orphan receptors from energy metabolism to cardiac hypertrophy.

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APPENDIX