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A Mechanistic Study of ERR α/γ Agonists for Treatment of Metabolic Dysfunction in Heart Failure

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by

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List of Abbreviations

AF1/AF2	activation function 1 and 2
ATP	adenosine triphosphate
BAF	bafilomycin A1
b.i.d	two times a day
BRDU	bromodeoxyuridine
C_2C_{12}	murine myoblast cell line
ChIP	chromatin immunoprecipitation
DBD	DNA binding domain
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ERR	estrogen related receptor
ERRE	estrogen related receptor response element
ETC	electron transport chain
FAO	fatty acid oxidation
FBS	fetal bovine serum
HEK293	human embryonic kidney cells
HFpEF	heart failure with preserved ejection fraction
HFrEF	heart failure with reduced ejection fraction
hiPSC-CM	human induced pluripotent stem cell-derived cardiomyocytes
i.p.	intraperitoneal injection
LBD	ligand binding domain
LBP	ligand binding pocket

MOI	multiplicity of infection
NCOR1	nuclear receptor corepressor 1
nM	nanomolar
NRVM	neonatal rat ventricular myocytes
OCR	oxygen consumption rate
OXPHOS	oxidative phosphorylation
PGC1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PK/PD	pharmacokinetic/pharmacodynamic
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
ROS	reactive oxygen species
SAR	structure activity relationship
TAC	transverse aortic constriction
TCA	tricarboxylic acid
TFEB	transcription factor EB
μM	micromolar

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McKenna Losby

Washington University in St. Louis

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Dedicated to those who have loved and supported me through this chapter of my life.

ABSTRACT OF THE DISSERTATION

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Professor Thomas Burris, PhD, Chair

Professor Brian Finck, PhD, Co-Chair

Heart failure is the leading cause of death in the United States and current therapeutic interventions fail to reverse the disease progression. In the failing heart, pathological cardiac remodeling leads to disease progression: a process characterized by hypertrophy, inflammation, fibrosis, and metabolic remodeling. By pharmacologically targeting the nuclear hormone receptors estrogen receptor-related receptor α and γ (ERRα and γ), it may be possible to reverse the array of metabolic pathways that are pathologically inhibited in the failing heart. Fatty acids are the primary fuel source of the adult heart that generate the ATP required for each contraction; however, in heart failure there is a shift in fuel utilization to anaerobic glycolysis. This leads to inefficient ATP production and exacerbates the progression of the failing heart. Genetic loss of function of either ERRα or γ leads to development of heart failure in mice due to a shift in fuel preference from fatty acids to glucose, which is reminiscent of the alterations observed in patients with heart failure. We have identified and characterized novel ERR agonists that can be used as pharmacological tools to

examine the potential beneficial effects of targeting the receptors to treat heart failure. Examination of gene expression changes induced by ERR agonists via RNA-sequencing in neonatal rat ventricular myocytes (NRVMs) revealed activation of many genes encoding enzymes in pathways such as tricarboxylic acid (TCA) cycle, fatty acid oxidation (FAO), oxidative phosphorylation (OXPHOS), and autophagy/mitophagy. We observed an increase in electron transport chair (ETC) proteins, mitochondrial content, activity, and respiration capacity with treatment of our novel ERR agonists. Additionally, a novel role for ERR in the autophagy-lysosome pathway was elucidated. Autophagy is the cells degradative and recycling pathway that is essential for physiological cardiac function and is dysregulated in the failing heart. NRVMs treated with ERR agonists show an increase in autophagic flux measured by markers such as the LC3 and p62 proteins. Importantly, the ERR agonist directly increases the expression of the transcription factor EB (TFEB), a master regulator of the autophagy-lysosome pathway. Upon ERR activation, we observed increased expression of TFEB target genes, highlighting the signaling cascade that leads to autophagy induction through ERR. In conclusion, targeting ERR is a promising potential therapeutic for improving heart function by both alleviating mitochondrial dysfunction and normalizing the deficit in autophagy that occurs in the failing heart.

Chapter 1: Introduction

1.1 Nuclear Receptor Protein Class

Nuclear receptors are transcription factors that are classically activated by binding to lipophilic ligands and regulating target gene expression. These receptors are structured with a highly conserved DNA binding domain (DBD) and a hydrophobic ligand binding domain (LBD), connected by a hinge region (**Figure 1.1**). The DBD has two zinc (Zn) finger motifs each with four cysteine residues, and the first Zn finger has a P-box containing five residues that are crucial to specificity of DNA binding.[1] The Zn fingers help maintain the DBD secondary structure that contain two alpha helices containing 66 amino acid residues.[2] The helix 12 in the LBD region contains an LXXLL motif that is responsible for promoting coregulator binding: a crucial aspect of transcriptional regulation. Nuclear receptors have two activation function domains (AF1 and AF2); AF1 is in the N-terminus and is a ligand-independent activation domain while AF2 is towards the C-terminus and is responsible for ligand-dependent transactivation function.[1] AF2 is not present in all nuclear receptors. For example, REV-ERB does not contain an AF2 domain, leading to the receptor functioning as a transcriptional repressor.[3]

Figure 1.1: Structure of nuclear receptors. A/B) Activation function 1 is ligand independent. C) DNA binding domain (DBD) interacts with DNA through zinc finger motifs. D) Hinge region is a flexible linker region between the DBD and LBD. E) Ligand binding domain (LBD) has a ligand binding pocket and region that interacts with coregulator proteins. F) Activation function 2 has ligand dependent function. This domain is not present in all nuclear receptors.

The 48 nuclear receptors are classified into seven subgroups (0-6) based on their sequence homology (**Table 1.1**). Subgroup 0 contains the nuclear receptors dosage-sensitive sex reversal-adrenal hypoplasia congenital critical region on the X chromosome, gene 1 (DAX1) and small heterodimer (SHP) that are unique due to their LBD resembeling coactivators, so they can interact with other nuclear receptors LBDs as a coactivator protein would.[4] Subgroup 1 binds lipophilic ligands and contains the nuclear receptors thyriod hormone receptor (TR), retinoic acid receptor (RAR), peroxisome proliferator-activated receptor (PPAR), reverse-Erb (REV-ERB), retinoid orphan receptor (ROR), farnesoid X receptor (FXR), liver X receptor (LXR), pregnane X receptor (PXR), and vitamin D receptor (VDR).[5] For a receptor to be termed "orphan", their natural ligand has not been identified. Adopted receptors were once orphans but since have had natural ligand identified.[6] Subgroup 2 contains the orphan receptors testicular receptor 2 (TR2), testicular receptor 4 (TR4), tailless homolog orphan receptor (TLX), photoreceptor-cell specific nuclear receptor (PNR), chicken ovalbumin upstream promoter-transcription factor (COUP-TF) and hepatocyte nuclear factor-4 (HNF4), and retinoid X receptor (RXR) which binds 9-cis retinoic acid.[5] Subgroup 3 binds to steroid hormones and contains androgen receptor (AR), progesterone receptor (PR), glucocorticoid receptor (GR), mineralocorticoid receptor (MR), and estrogen receptor (ER), and due to their structural similarity, includes the orphan estrogen-related receptors (ERR).[7] Subgroup 4 contains orphan receptors nerve growth factor 1B (NGF1-B), nuclear receptor related 1 (NURR1) and neuron-derived orphan receptor 1 (NOR-1).[5] Subgroup 5 contains steroidogenic factor 1 (SF-1) and liver receptor homolog-1 (LRH-1), which are classified to be orphans, but data suggest they bind phospholipids.[8] Lastly, subgroup 6 contains the orphan receptor germ cell nuclear factor (GCNF) that is important for embryonic development.[9]

Subgroup	Abbreviated name
0	DAX1, SHP
1	TR, RAR, PPAR, REV-ERB, ROR, FXR, LXR, PXR, VDR
2	TR2, TR4, TLX, PNR, COUP-TF, HNF4, RXR
3	AR, PR, GR, MR, ER, ERR
4	NGF1-B, NURR1, NOR-1
5	SF-1, LRH-1
6	GCNF

Table 1.1: The nuclear receptor superfamily is divided into seven subgroups (0-6). These subgroups are defined by sequence homology.

In their inactive state, nuclear receptors may either reside in the cytoplasm or the nucleus, depending on the specific nuclear receptor in question. The nuclear receptors that reside in the cytoplasm in their inactive state are bound to heat shock proteins and upon ligand binding are released from the heat shock proteins due to conformational changes.[10,11] The now active nuclear receptor translocates to the nucleus and undergoes dimerization. Alternatively, nuclear receptors that reside in the nucleus are bound to their respective response element, but kept in an inactive state due to corepressor proteins (**Figure 1.2A**).[2,12] SMRT and NCoR are corepressor proteins that interact with the LBD of many nuclear receptors to supress transcriptional activity.[13] Upon ligand binding the nuclear receptor undergoes a conformational change and then binding of coactivator proteins (**Figure 1.2B**). Nuclear receptor coactivators (NCOA1-3), SMARC, and PGC1α are exacmples of a few common coactivator proteins that activate transcriptional activity of nuclear receptors through interacting with the LBD and inducing a conformational change of the

receptor.[14] Nuclear receptors may bind to their respective DNA response element in monomeric or dimeric form. Those that bind in dimeric form may bind as homodimers or heterodimers with the binding partner retinoid X receptor (RXR). Nuclear receptor response elements are structured in three general classes: direct-repeats, inverted repeats, or extended monomeric sites.[15] The direct or inverted repeats may have zero to six spacer nucleotides separating the half-site.[16] These response elements are typically found upstream of the transcription site in the promoter region of the gene.

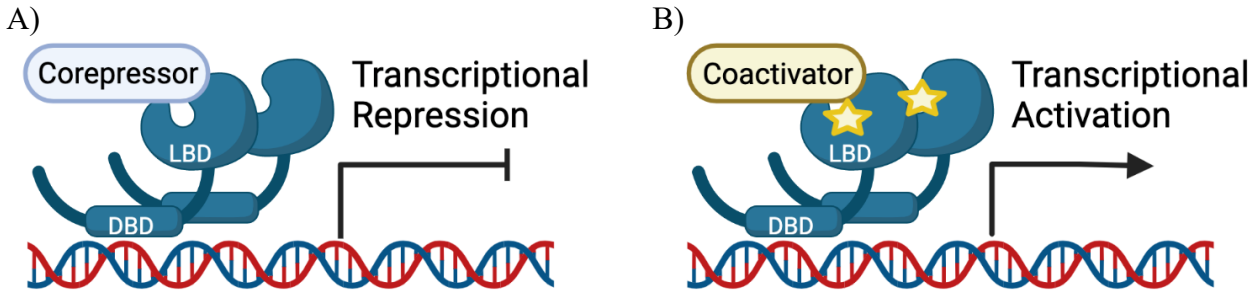

Figure 1.2: Regulation of nuclear receptors by coregulator proteins. A) Corepressor proteins bind to nuclear receptors and inhibit transcription. B) Coactivator proteins bind to nuclear receptors and activate transcriptional regulation.

Nuclear receptors can have an impact on cellular signaling beyond their traditional transcriptional response described above. Nuclear receptors can have non-genomic effects that have been more recently studied such as regulation of secondary messengers, intracellular calcium levels, kinase and phosphatase activity, and more.[17] Additionally, nuclear receptors can engage in tethered trans-repression, a process by which activated nuclear receptors interact with other transcription factors and repress their signaling pathways.[18] Two well described examples of nuclear receptor tethering occur with activator protein-1 (AP-1) and nuclear factor-κB (NF-κB),

and this response can be dependent on nuclear receptor corepressors.[19] The field is expanding for studying nuclear receptors action on non-genomic responses and tethered trans-repression.

Small molecule therapeutics can be designed to target the ligand binding pocket of nuclear receptors. These small molecules may be agonists, antagonists, or inverse agonists, all of which elicit different biological responses. Agonists are molecules that bind in a similar manner to endogenous ligands and produce a similar response. Agonist binding typically results in a conformational change that recruits coactivators to enhance transcription of target genes. Of note, some nuclear receptors are transcriptional repressors- such as REV-ERB- and upon treatment with an agonist decreases expression of target genes.[20] Alternatively, therapeutics can be designed as antagonists, which block the binding of natural ligand and prevent the endogenous response. Lastly, inverse agonists reduce the basal response of the receptor and decrease target gene transcription.[21] Small molecules are designed with rational drug design, docking methods, and structure activity relationship (SAR) optimization. If the natural ligand is known, modifications to its structure may be made to increase affinity to the LBD. Docking methods help elucidate key residues that are involved in ligand binding, so ensuring those interactions are kept with novel compounds may help in successful design.

Nuclear receptors are excellent therapeutic targets with high clinical success rates. They regulate numerous biological pathways- such as metabolism, growth, development, circadian clock, and immune system- and are expressed in a variety of tissue and cell types.[10] This makes this protein class diverse in the multitude of diseases that may be targeted. A few examples of FDA

approved drugs that target nuclear receptors are tamoxifen (breast cancer), rosiglitazone (anti-diabetic agent), tretinoin (dermatologic treatment), and dexamethasone (anti-inflammatory).[22,23,24] There are many clinical trials underway targeting nuclear receptors for a variety of diseases.

1.2 The Nuclear Receptor Estrogen Related Receptor

The estrogen related receptors (ERRs) are orphan nuclear receptors in the NR3B subgroup. ERRα and ERRβ were first discovered by Ronald Evans' lab in 1988. When performing a cDNA hybridization screen on ERα, they found novel proteins with high sequence similarity to other steroid hormone receptors.[25] The ERRs cannot bind β-estradiol yet were named as such due to having high homology with the estrogen receptor, its closest related receptor in the NR superfamily. ERRγ was discovered in 1998 by Janos Sumegi's lab when studying Usher type IIa disease. In a screen of fetal brain cDNA library they identified a novel sequence that had high similarity to ERRα and ERRβ, naming it ERRγ.[26]

The ERRs are orphan receptors, since the natural ligand is unknown, however, they are constitutively active, demonstrating an elevated level of basal activity even without any ligand present. ERRs transcriptional activity is regulated by the relative levels of coregulator proteins like PGC1α/β, NCOR1, and others.[27] These co-regulator proteins can interact with the LBD of ERR to switch it into an active or inactive conformation. PGC1α specifically plays an important role in ERRs transcriptional regulation due to its ability to induce expression of ERRs and also interact with the receptor to induce activating conformational changes.[28] PGC1α is sometimes referred to as a "protein ligand" for ERR due to its role in the constitutive activity of this orphan receptor.

The ERRs may bind as a monomer, homodimer, or heterodimer to a DNA response element, called ERRE, with a specific nucleotide sequence- TCAAGGTCA.[29,30,31] The ligand binding pocket (LBP) of ERRα and ERRγ is approximately 100 and 220Å^3, respectively, which are the smallest of the nuclear receptor class compared to an average sized LBP volume of ERα 450Å^3 and the largest PPARs 1400Å^3 size.[29,32,33] This extremely small ligand binding pocket size has led to difficulty in small molecule ligand development.

The ERRs are expressed in tissues with high energy demands like the brain, heart, muscle, and brown adipose tissue. ERRα is ubiquitously expressed and at higher levels than the other two isoforms.[34] ERRα is known to directly regulate expression of genes involved in lipid and carbohydrate metabolism.[35] ERRα is involved in adapting to environmental stresses, such as cold exposure, through enhancing thermogenesis by regulating OXPHOS in brown adipose tissue.[36] Upon exercise, ERRα expression is increased, and its gene network activated, showing importance of the receptor in muscle function and fitness.[37] ERRγ is important for energy homeostasis, learning and memory in the brain.[38] ERRβ is highly expressed during development and has involvement in placental formation.[39] ERRα/γ are the dominant expressed isoforms in the mature organism, so will be the focus of most of the discussion herein.

Given the expression patterns of ERR and effects in various tissues mentioned above, it has been found the ERRs regulate the expression of numerous genes involved in pathways such as fatty acid oxidation (FAO), TCA cycle, oxidative phosphorylation (OXPHOS), and mitochondrial

biogenesis.[40,41] The first discovered ERRα target gene, *Acadm*, encodes the enzyme medium-chain acyl coenzyme A dehydrogenase (MCAD), that catalyzes a key step in mitochondrial beta-oxidation.[42] Another key regulator of mitochondrial metabolism that is directly regulated by ERRs is pyruvate dehydrogenase kinase 4 (*Pdk4).*[40] The mitochondrial TCA cycle generates reducing equivalents that can be used by the electron transport chain to generate ATP, and the ERRs regulate the transcription of many enzymes of this cycle: *Cs, Aco2, Ogdh, Sdhb* genes to name a few.[40] ERR also regulates expression of genes such as *Cox6A1, Cox7A2, Atp5B,* and *Uqcr1* that encode the various complexes involved in oxidative phosphorylation for the generation of ATP.[40] ERRs regulate glucose metabolism and anaerobic glycolysis by activating the expression of glucose transporter (*Slc2A1),* hexokinase 2 *(Hk2),* glyceraldehyde dehydrogenase *(Gapdh),* and enolase 1 *(Eno1)*.[43] ERRα/γ target genes largely overlap; making the identification of isoform specific target genes a difficult task.

There has been interest for many years in targeting the ERRs with synthetic compounds for a multitude of diseases. ERR inverse agonists were relatively straight-forward to identify. The first ERRα inverse agonist, XCT790, was reported by Busch et. al. in 2004, and by other groups was found to inhibit breast cancer cell growth, increase mitochondrial ROS production, while diminishing mitochondrial abundance and membrane potential.[44,45] XCT790 has an IC_{50} of 0.37 μM for ERRα as verified in a luciferase reporter assay. However, upon further study, it was found that XCT790 induces mitochondrial uncoupling and depletion of ATP concentrations, independently of ERR, confounding the use of this compound as a tool to study ERR biology.[46] The first ERRβ/γ agonist, GSK4716, was reported by Zuercher et. al. in 2005 and increased

PGC1α expression and increase mitochondrial function.[47,48] Our lab developed a selective ERRα/γ inverse agonist named SLU-PP-1072 that was derived from GSK4716 scaffold. SLU-PP-1072 has an IC_{50} of 4.8 μM for ERRα and 0.9 μM for ERRγ verified in a luciferase reporter assay and proved to be beneficial for treatment of prostate cancer.[49] Compound 29 (C29) was discovered to be a novel ERRα inverse agonist with an IC_{50} of 0.6 μM for ERRα with the potential to be a treatment for diabetes.[50] Origionally designed as an estrogen receptor inhibitor, 4–hydroxy tamoxifen was discovered to also be a ERRγ inverse agonist.[51] A novel ERRγ inverse agonist called GSK5182 was designed as an analog to 4-hydroxy tamoxifen.[52] GSK5182 has an IC_{50} of 0.079 μM for ERRγ. There are several ERR inverse agonists that can be used to study the receptors' mechanism of action, however the availability of ERR agonists are limited.

ERR agonists, particularly ERRα agonists, were very difficult to identify. The reason for the difficulty was twofold: the small size of the ligand binding pocket and the constitutive activity of the receptors. As previously mentioned, ERR has one of the smallest LBPs of the nuclear receptor class, so it was thought that the LBP could not accommodate a synthetic ligand, however, it was found that the pocket is dynamic and able to bind small molecule ligands. Secondly, the constitutive activity of ERR made some investigators believe the activity could not be enhanced further, which has since been disproved. A novel ERRα agonist called JND003 was described by Ding et. al. in 2022 as a potential therapeutic for nonalcoholic fatty liver disease and insulin resistance.[53] Through use of genetic and pharmacological models, they showed decreased steatosis and increased insulin sensitivity, validating targeting ERRα for these diseases. JND003 has an EC_{50} of 2.7 μM for ERRα, which was verified in a luciferase reporter assay.

As mentioned above, there are several potent ERR inverse agonists, but there is an unmet need for potent ERR agonists. In collaboration with John Walker at Saint Louis University, the Burris lab identified the first ERRα/γ agonists called SLU-PP-332 and SLU-PP-915 (**Figure 1.3A**). The potency of these compounds was tested in an assay wherein expression plasmids for full length ERRα, β, and γ were co-transfected with a luciferase reporter construct under the control of an ERR response element (**Figure 1.3B**). SLU-PP-332 had an EC_{50} of 98 nM with ERRα, 230 nM ERRβ, and 430 nM ERRγ making the compound slightly more alpha selective. SLU-PP-915 had EC_{50} values of approximately 400 nM for each isoform making the compound a pan-agonist. To test the selectivity, both compounds were run in a nuclear receptor specificity panel that comprehensively tests activity towards other nuclear receptors and were found to have selectivity with ERR (**Figure 1.3C**). Using molecular modelling, SLU-PP-915 was docked in the ERRα LBP and found to have interactions with residues in the pocket showing that the compound fits in the pocket in a thermodynamically favorable conformation (simulations for SLU-PP-332 were performed, but data not shown) (**Figure 1.3D**).

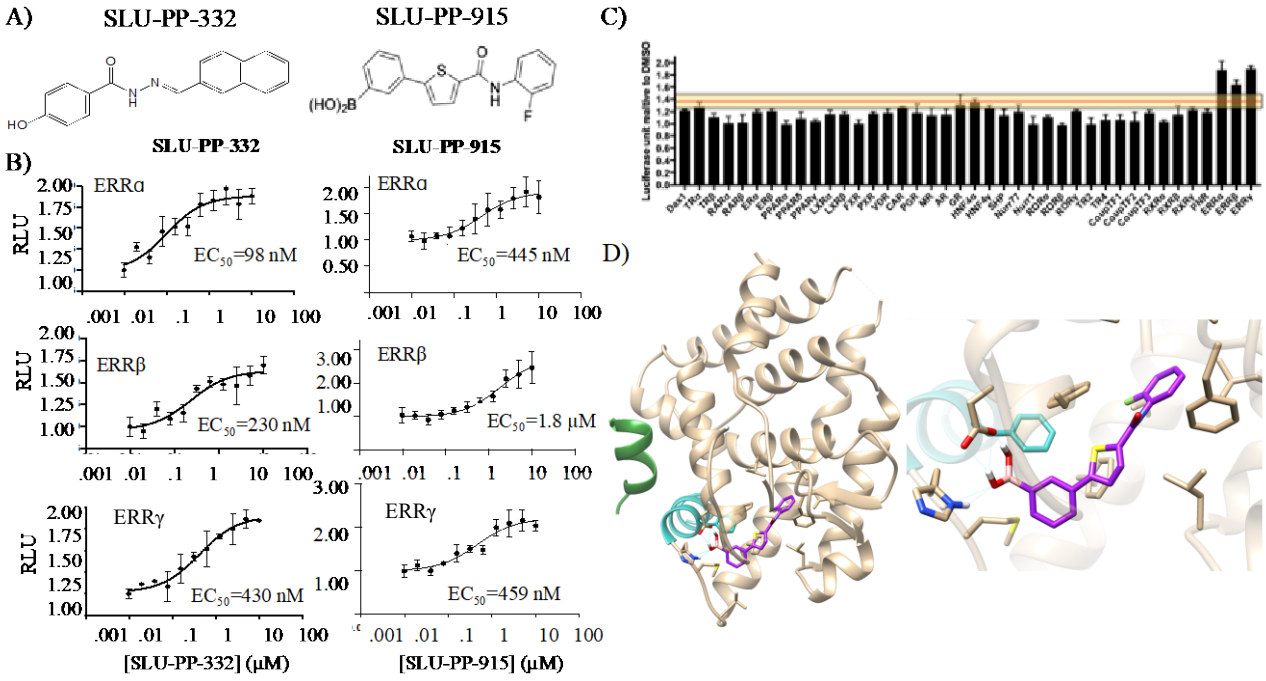

Figure 1.3: Characterization of novel ERR agonists. A) Compound structures of SLU-PP-332 (left) and SLU-PP-915 (right) are displayed. B) Luciferase reporter assay in HEK293T cells with full length ERR α, β, or γ show dose response dependency of the compounds with ERR activity. C) Nuclear receptor specificity panel ran in HEK293 cells for SLU-PP-332 (10 μM) shows selectivity of this compound for ERR α, β, and γ over the other nuclear receptors. D) SLU-PP-915 docked in ERR α LBP (PDB: 7E2E). Molecular modelling and pictures performed and generated by Lamees Hegazy.

1.3 Heart Failure Etiology and Current Treatments

The human heart is a specialized organ that pumps blood through our body so that our cells can get the oxygen they need to survive. The heart is composed of four chambers: right atrium, right ventricle, left atrium and left ventricle. Deoxygenated blood flows into the superior vena cava to the right atrium, then the right ventricle, and out through the pulmonary valve and pulmonary artery to the lungs. The lungs oxygenate the blood and send it back to the heart though the pulmonary vein into the left atrium, then left ventricle, and out of the heart through the aortic valve

and aorta to the rest of the body.[54] Each beat of the heart is crucial and takes an enormous amount of energy to sustain continuously.

The heart contains a mixed population of cells consisting of cardiac fibroblasts, cardiomyocytes, smooth muscle cells, and endothelial cells. Cardiac fibroblasts are important for maintaining the structure of the heart by secreting the extracellular matrix (ECM) and are found throughout the heart.[55] In heart disease, cardiac fibroblasts are the cells that mediate the development of fibrosis due to the production of collagen and fibronectin fibers that led to myocardial stiffening.[56] Smooth muscle cells are responsible for controlling blood pressure and blood flow.[57] Endothelial cells aid in blood flow by controlling vascular constriction and relaxation, leukocyte trafficking, and angiogenesis.[58] Lastly, cardiomyocytes are involved in generating contractile force and due to their high mitochondrial density, generation of ATP and other high energy phosphates.[59]

Cardiomyocytes undergo a metabolic transition in fuel utilization to a more oxidative profile when they differentiate from fetal to postnatal state: a process that is controlled by ERRγ.[60] The prenatal heart utilizes anaerobic glycolytic metabolism in the early phases of development.[61] The transition to the mature heart coincides with a change in fuel utilization to oxidative metabolism because the workload and substrate availability of the organism changes.[61] Glucose undergoes glycolysis to generate pyruvate and along the way generates just two molecules of ATP. Pyruvate then enters the mitochondria to be oxidized to acetyl-CoA.[62] Acetyl-CoA enters the TCA cycle to generate three molecules of NADH and one molecule of FADH2.[63] These reducing

equivalents are oxidized by the electron transport chain to generate ATP. Fatty acids are converted to fatty acyl-CoA, through fatty acyl-CoA synthetase, which can enter the mitochondria through a membrane protein transport system catalyzed by CPT1 and CPT2.[64] Fatty acyl-CoAs undergo beta oxidation to generate two acetyl-CoA molecules per turn of the beta oxidation pathway. Here, the pathway converges with pyruvate oxidation in also producing acetyl-CoA, which enters the TCA cycle to produce reducing equivalents for the OXPHOS pathway. The adult heart utilizes about 70-90% fatty acids to generate cardiac ATP, while approximately 1 mM of ATP is consumed per second by the working heart.[65,66] The high rates of ATP production and utilization emphasize the importance of this process being functional and efficient to maintain cardiac homeostasis.

Heart failure impacts over 6.2 million adults in the United States.[67] In the failing heart, stroke volume- volume of blood pumped out of the ventricle per contraction- and cardiac output- the rate of blood volume pumped by the heart- are decreased, which leaves the body and its organs in an oxygen deficiency.[68] This condition has many contributing causes including ischemic heart disease, atherosclerosis, high blood pressure, diabetes, and obesity that led to cardiac remodeling, cardiac dysfunction, and ultimately heart failure.[69] Pathologic cardiac remodeling is characterized by hypertrophy, inflammation, cardiomyocyte death, fibrosis, and metabolic remodeling.[70] One of the main contributors to heart failure is cardiac metabolic dysfunction. In both rodent models and human patients with heart failure, there was observation of decreased fatty acid utilization and an increase in glycolytic metabolism.[71] The reason for alterations in cardiac metabolism can be contributed to the failing heart having an energy deficit due to dysregulated mitochondrial function and temporarily compensates for this by increasing ATP production from glycolytic metabolism.[72]

However, this may become maladaptive and lead to further disease progression with cardiac hypertrophy and remodeling.

In heart failure, gene expression patterns are altered for fatty acid utilization, TCA, and mitochondrial oxidative phosphorylation. Expression of genes encoding key steps in fatty acid uptake and oxidation, such as *Cpt1b*, *Mcd,* and *Pdk4* are reduced in the failing heart, while genes encoding key steps in glucose uptake and glycolysis are upregulated.[73] Precursors of the TCA cycle are rerouted towards hypertrophic growth, resulting in a shift to higher rates of anaplerosis to sustain the TCA cycle.[74] Anaplerosis is the process of adding intermediates to the TCA cycle and when the TCA intermediates are depleted, rates of anaplerosis from glutamine and other nutrients is increased. Evidence of increased anaplerotic flux has been detecting in cardiac hypertrophy.[75] Proteins that make the OXPHOS complexes in the ETC are decreased in the failing heart, along with evidence of abnormal mitochondrial morphology.[74] Taken together, targeting the depressed function of FAO, the TCA cycle, and OXPHOS could be a viable therapeutic intervention.

The two types of heart failure are broadly defined by whether left ventricular ejection fraction is preserved or reduced: heart failure with preserved ejection fraction (HFpEF) and heart failure with reduced ejection fraction (HFrEF). The ejection fraction (EF) is the amount of blood exiting the heart upon each beat, measured as a percentage.[76] The ejection fraction is calculated by the ratio of stroke volume and end-diastolic volume.[77] An ejection fraction above 55% is considered normal and HFrEF is characterized as an ejection fraction below 40%. Patients may

seek medical advice due to fatigue, reduced exercise capacity, swelling of ankles, and shortness of breath.[78] HFrEF is primarily caused by volume overload and systolic dysfunction that over time causes injury to the myocardium.[79] Murine models of HFrEF include transverse aortic constriction (TAC) to study pressure overload, coronary artery ligation for myocardial infarction, aortocaval fistula to model volume overload, and several models where HFrEF is induced by small molecules.[80] In contrast to HFrEF, with HFpEF the EF is typically near "normal" (above 50%), but is still associated with cardiac hypertrophy. HFpEF is a complex disease with substantial heterogeneity among patients, making treatment challenging. HFpEF is typically associated with comorbidities such as obesity, type 2 diabetes mellitus, chronic obstructive pulmonary disease, hypertension, and other metabolic syndromes.[81] Additionally, HFpEF is associated with aging and, interestingly, women are at higher risk for developing the disease. This form of heart failure has less therapeutics intervention that may be caused by inadequate model systems to study the complex disease. Unlike HFrEF models mentioned above, many HFpEF models, such as high fat diet, aging, angiotensin II infusion, and leptin deficient or leptin receptor deficient (ob/ob and db/db) mice, do not fully recapitulate the disease.[82] It has been proposed to use a combination of mentioned models to replicate the disease phenotype more extensively. According to a recent study, the mortality rates between HFrEF and HFpEF are not significantly different; however, all heart failure patients have greater than 50% mortality rate in the 5 years after diagnosis emphasizing the unmet need for novel therapeutics.[83,84]

The two main types of heart failure have different therapeutic approaches for treatment. HFrEF treatment includes neurohormonal inhibitors such as angiotensin converting enzyme (ACE) inhibitors, angiotensin II receptor blockers (ARBs), beta-blockers, and mineralocorticoid

receptor antagonists (MRAs).[85] The main action of all of these agents is to reduce blood pressure. Treatment of HFpEF is not as straight-forward and there has not been any indication of decreased mortality of patients with HFpEF with current pharmacological interventions.[86,87] Therapies such as diuretics, SGLT2 inhibitors, ARBs, and MRAs may be used depending on patients' symptoms.[86] Although treatments are available, heart failure still causes about 1 in every 4 deaths in the United States, emphasizing the need for improved therapeutics.[88] While traditional treatments focus on improving neurohormonal stress and hemodynamics, there are no FDA approved drugs that directly target the maladaptive metabolic changes in the myocardium.

1.4 ERR as a Therapeutic Target for Heart Failure

Genetic knockout studies have provided insight on the function of ERR in heart homeostasis (**Table 1.2**). Whole body ERRα KO mice display bioenergetic defects and failure to adapt to cardiac pressure overload, while ERRγ KO is lethal within 48 h of birth highlighting the importance of ERRγ role in metabolic shift to oxidative metabolism.[89,90,91] Whole body ERRβ knockout results in embryonic lethality suggesting an essential role for this receptor in embryonic development.[92] Cardiac specific ERRβ knockout die within the first months of life and display heart failure phenotypes.[93] Interestingly, cardiac specific ERRα have normal cardiac function and cardiac specific ERRγ have decreased expression of mitochondrial related genes, but no substantial cardiac alterations, suggesting a possible functional overlap between the isoforms.[93] In support of this, cardiac specific ERRα/γ KO mice exhibit heart failure with dysregulated metabolic, contractile, and conductive function highlighting the important role of ERRα and γ in cardiac homeostasis.[93]

KO	Phenotype
Whole body ERRα	Pressure overload-induced pathological remodeling
Whole body ERRβ	Embryonic lethal
Whole body ERRγ	Premature death at 48hrs
Cardiac ERRα	Normal cardiac function
Cardiac ERRβ	Dilated cardiomyopathy and sudden death at 10 months of age
Cardiac ERRγ	Decrease expression Mt genes, no gross cardiac alterations
Cardiac ERRα/γ	Dies within the first month of age with cardiomyopathy and heart failure

Table 1.2: Knock out studies for each of the ERR isoforms, in both whole body and cardiac specific deletions, with their respective phenotypes. These studies show evidence for the important function of ERR in the heart.

ERR expression decreases in the failing heart, so targeting ERR through small molecule agonists would aim to normalize the physiological activity of the receptor. This hypothesis is supported through cardiac ERR KO studies discussed previously. However, it should be noted that cardiac overexpression of ERR has been described to have detrimental effects. Mice with cardiac specific ERRγ overexpression displayed early lethality compared to WT littermates with cardiomyopathic remodeling including increased hypertrophy, fibrosis, and apoptosis.[94] This observation was further confirmed through pharmacological modulation of ERRγ with the inverse

agonist GSK-5182. In cardiomyocytes treated with GSK-5182 it was found that cardiac hypertrophy induced by phenylephrine was blocked, suggesting ERRγ repression suppresses hypertrophy.[95] However, overexpression of many transcriptional regulators that exceedingly increase metabolism can cause cardiomyopathy: such as PGC1α, PPARα, and others.[96,97] It is clear that overexpression and knockout of ERR causes cardiac dysfunction, but activating the receptor with a small molecule agonist to study the impact on cardiac function has not been explored. I hypothesize that normalizing ERRs activity to physiological levels in the failing heart will be therapeutically beneficial due to the transcriptional regulation ERR has on metabolic pathways that are decreased in the disease state.

1.5 Hypotheses Addressed in this Thesis

This thesis addresses a novel approach to treat heart failure by targeting mitochondrial dysfunction and autophagy deficits through pharmacologically targeting the estrogen related receptor (**Figure 1.4**). The rationale for this hypothesis stems from the knowledge of the role ERRs play in transcriptionally modulating metabolic pathways such as fatty acid oxidation, TCA cycle, and OXPHOS: processes that are critical for mitochondrial function. The metabolic dysfunction that leads to pathological cardiac remodeling is caused by a shift in fuel utilization from oxidative to glycolytic metabolism. This altered metabolic state leads to mitochondria that do not function properly nor produce adequate ATP for the cardiomyocyte. The pathological remodeling that occurs due to these deficits leads to reduced capacity to perform vital cellular tasks such as autophagy. Autophagy is the cells conserved degradative pathway that is necessary to maintain cellular homeostasis. With reduced autophagic capacity the cardiomyocyte has increased

accumulation of damaged proteins and organelles, thus creating a toxic environment that ultimately leads to apoptosis.

Chapter 2 of this thesis describes the work on ERR agonists SLU-PP-915 and SLU-PP-332 and their effects on mitochondrial function relevant to heart failure. I hypothesized that ERR agonists will reverse an array of metabolic pathways that are deactivated in the failing heart. Specifically, I investigated gene and protein expression of key ERR target genes that are involved in TCA, OXPHOS, and FAO pathways. Then, I performed functional assays to assess fatty acid oxidation rates, quantifying mitochondrial content, and mitochondrial function.

Chapter 3 of this thesis focuses on ERRs role in cardiac autophagy. Impaired autophagy and mitochondrial dysfunction are strongly linked, particularly in heart failure, and both negatively contribute to disease progression. I hypothesized that ERR agonists will increase autophagic flux in cardiomyocytes through activation of TFEB. First, this hypothesis is assessed through quantification of gene and protein expression of key autophagy players. Next, I performed functional assays of autophagy by measuring autophagic flux. Our observation that ERR regulates autophagy pathway led to the discovery of a novel ERR target gene, TFEB, which marries the mitochondrial health and autophagy pathway. ERR regulation of TFEB was characterized through target gene expression, luciferase reporter assay, and immunofluorescent techniques.

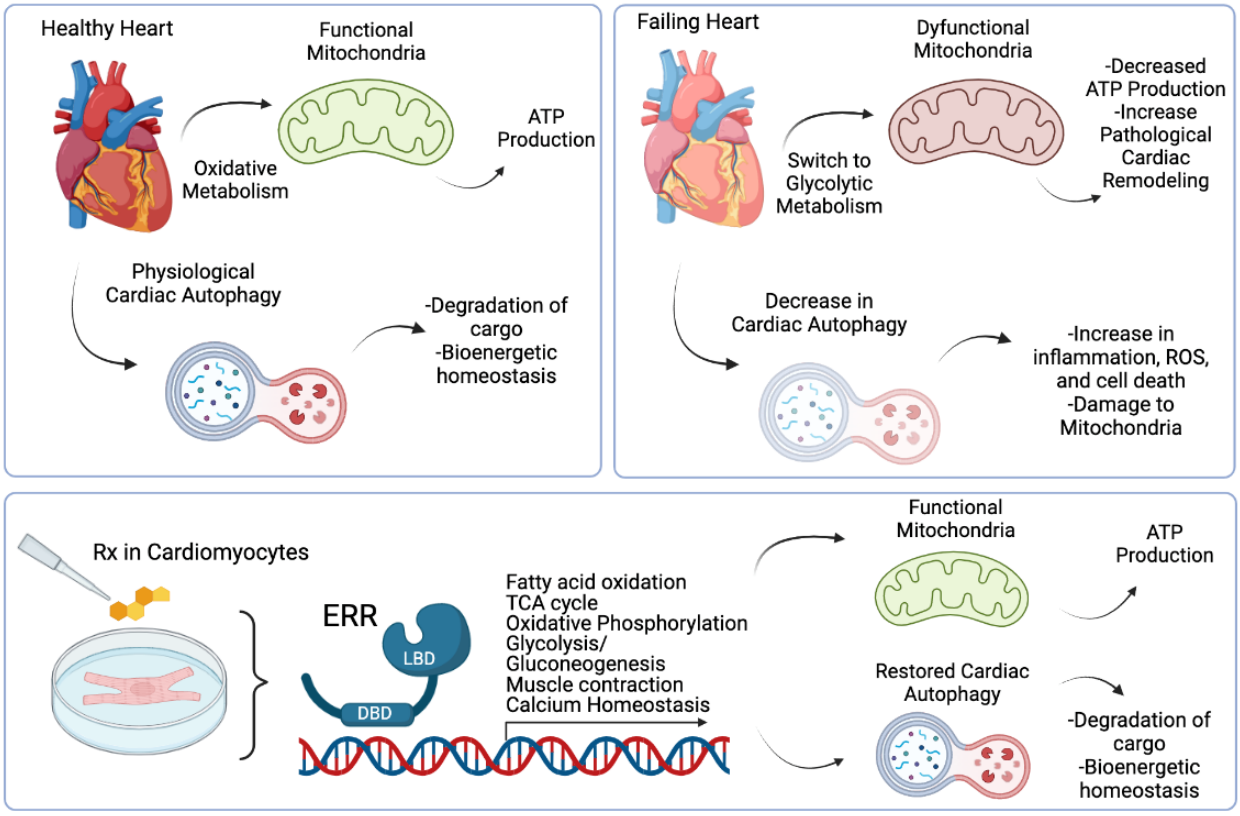

Figure 1.4: The healthy heart relies on oxidative metabolism and physiological cardiac autophagy to generate ATP and fuel the cardiomyocytes. In heart failure there is a switch in fuel utilization to glycolytic metabolism, which leads to dysfunctional mitochondria and further propagates the pathological cardiac remodeling. There is a decrease in cardiac autophagy that leads to damaged mitochondria and cell death. By treating cardiomyocytes with ERR agonists that increase a variety of metabolic pathways, it is hypothesized that the mitochondria will return to a functional state and generate the necessary ATP production to alleviate pathological effects of heart failure and restore cardiac autophagy to lead to bioenergetic homeostasis.

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Chapter 2: ERR Agonists Increase Mitochondrial Function

2.1 Preface

Parts of this chapter are modified from a paper published on the bioRxiv preprint server (https://doi.org/10.1101/2022.02.14.480431). The work outlined in this chapter was done in collaboration with myself, Cyrielle Billon, Matthew Hayes, Weiyi Xu, Thomas Burris, and Lilei Zhang. In this chapter, I am responsible for designing, executing, and analyzing data of experiments for i) cytotoxicity studies, ii) qPCR gene expression assays, iii) western blotting assays, iv) fatty acid oxidation assays, v) flow cytometry with MitoTracker assay, and vi) mitochondrial content quantification assay. WX is the first author of the mentioned paper and did a majority of manuscript writing and experimental assays. TB and LZ are both PIs on this paper and responsible for designing and coordinating research in this collaboration. CB and MH assisted in experimental design, performed experiments, and analyzed data. Specifically, data shown in this chapter that were performed by others include i) RNA-sequencing and ii) seahorse OCR assay.

2.2 Abstract

Heart failure impacts over 6.2 million adults in the United States and current treatments are unable to ameliorate the disease. We aim to reverse the dysregulation of an array of metabolic pathways that are pathologically inhibited in the failing heart through pharmacologically targeting the nuclear hormone receptor estrogen receptor-related receptor (ERR). The healthy adult heart

relies on fatty acid oxidation (FAO), lactate/pyruvate oxidation, and downstream oxidative phosphorylation (OXPHOS) for generation of ATP. However, in the failing heart there is a metabolic shift towards glycolysis for ATP production, which has been shown to coincide with decreased expression of ERRs. Additionally, ERRα/γ plays a significant role in physiological cardiac function as seen by both ERRα and γ knockout mice displaying heart failure phenotypes. Examination of gene expression changes induced by ERR agonists via RNA-sequencing in neonatal rat ventricular myocytes (NRVMs) reveals increased expression of many genes in pathways that are pathologically deactivated in failing heart, including the TCA cycle, FAO, OXPHOS, and autophagy/mitophagy. We observe an increase in ETC proteins, mitochondrial content, activity, and respiration capacity. Pharmacologically targeting ERR has therapeutic potential for heart failure through improving mitochondrial function.

2.3 Introduction

During heart failure, the metabolic shift in fuel utilization propagates disease progression. In the healthy heart, cardiomyocytes are most efficiently fueled by fatty acids for mitochondrial generation of ATP. In the failing heart, glycolytic metabolism is enhanced, in association with mitochondrial dysfunction. This mitochondrial dysfunction leads to decreased ATP production by the OXPHOS complex, increased ROS generation, and activation of signaling cascades in the cardiomyocyte that may lead to apoptosis. The heart attempts to resolve this issue through cardiac remodeling; however, this remodeling can eventually further propagate disease progression. Activating ERR to enhance transcriptional regulation of genes involved in mitochondrial

metabolism and ATP production may reverse the metabolic dysfunction and further remodeling by the cardiomyocytes during heart failure.

The mitochondrion is an extremely unique and important organelle. Since mitochondria are derived from α-proteobacteria that entered the eukaryotic cell via endocytosis billions of years ago, they have their own distinct DNA.[1] Mitochondria are double membrane organelles separated from the cytosol by an inner and outer mitochondrial membrane. The outer membrane lacks membrane potential due to its porous nature; however, the inner membrane has a tightly regulated electrochemical potential that is required for oxidative phosphorylation.[2] These membranes separate the mitochondria into the intermembrane space and matrix compartment. The inner membrane is invaginated to increase surface area and form structures called cristae that contain most of the mitochondrial respiratory chain complexes.[3] The OXPHOS machinery consists of five complexes that catalyze the last steps in ATP production: Complex I- NADH dehydrogenase, Complex II- succinate dehydrogenase, Complex III- cytochrome c reductase, Complex IV- cytochrome c oxidase, and Complex V- ATP synthase. The matrix has a higher pH due to the ETC pumping H^+ to the inner membrane space that creates an electrochemical potential and favorable Gibbs free energy to facilitate Complex V's activity.[4]

Several diseases are caused by mitochondrial dysfunction or have secondary effects that cause mitochondrial dysfunction. Mitochondria communicate with the rest of the cell through signaling pathways such as cytochrome c release, reactive oxygen species (ROS), AMPK activation, and mtDNA release.[5] During stress or damage to the mitochondria, cytochrome c can

be released and induce the cell death apoptosis pathway.[6] An increase in ROS in the mitochondria causes membrane pores to open and release ROS to maintain normal levels in the organelle. However, ROS can be toxic to the cell and induce DNA damage.[7] AMPK activation is an important regulator of mitochondrial health by promoting mitochondrial biogenesis or mitophagy depending on energetic conditions.[8] Upon cellular stress or damage, mtDNA can be released from the mitochondria and trigger immune response.[9] Collectively, these alterations in homeostatic mitochondrial function can lead to neurodegenerative, metabolic, and cardiovascular disorders. In the context of the heart disease, increasing the transcriptional regulation of ERR target genes through small molecule agonists may normalize the deficits seen in metabolic pathways and reverse mitochondrial dysfunction.

Prior data from the Burris lab characterizing the ERR agonists SLU-PP-332 and SLU-PP-915 gave insight on the utility of targeting ERRs to treat metabolic dysfunction, leading to my thesis work focusing on heart failure. In a C_2C_{12} murine myoblast cell line it was observed that upon treatment of SLU-PP-332, MitoTracker Red signal, a mitochondrial marker of function, was significantly increased compared to control.[10] In these C_2C_{12} cells, SLU-PP-332 also increased the maximum mitochondrial respiratory capacity as analyzed by Seahorse XF analyzer. After promising preliminary data *in vitro*, the lab decided to perform pharmacokinetic (PK) studies and an *in vivo* experiment on a transverse aortic constriction (TAC) model of heart failure.[11] The PK data showed 50 mg/kg b.i.d. i.p. (twice a day by intraperitoneal injection) would give ample drug exposure. Then, after experimental TAC, mice were treated with SLU-PP-332 for 6 weeks, which resulted in an impressive restoration of ejection fraction towards values observed in mice undergoing sham surgery. SLU-PP-332 treated TAC mice also displayed decreased cardiac

fibrosis and an increase in mitochondrial DNA number. Electron microscopy showed that cardiac mitochondria from TAC mice treated with vehicle lost their distinct cristae structure, while mitochondria from TAC mice treated with SLU-PP-332 exhibited a crisp and well-defined cristae, similar to the sham group. Taken together, these preliminary data prompted me to examine the mechanism by which activating ERR improves mitochondrial function and heart metabolism.

2.4 Results and Discussion

2.4.1 ERR Agonists Induce Expression of ERR Target Genes *in vivo*

To assess the effects of these compounds on ERR target gene expression, wild type mice were dosed at 50 mg/kg and 25 mg/kg respectively, b.i.d. for seven days. RNA was extracted from heart tissue and expression of target genes assessed via qRTPCR. Both drugs activated ERR target gene expression of genes involved with mitochondrial biogenesis, fatty acid oxidation, TCA cycle, and oxidative phosphorylation (**Figure 2.1**). For all genes, SLU-PP-915 increased expression more dramatically than SLU-PP-332, which may be due to SLU-PP-915 exhibiting a more balanced dual ERRα/γ agonist and SLU-PP-332 targeting ERRα more selectively. In addition, SLU-PP-332 has a shorter half-life than SLU-PP-915, so it may elicit a more acute response while SLU-PP-915 has a more sustained response. Animals did not display any negative side-effects and tolerated the dosing. With validation of ERR target gene expression *in vivo,* the next step was to validate SLU-PP-332 and SLU-PP-915 in the *in vitro* cardiomyocyte model.

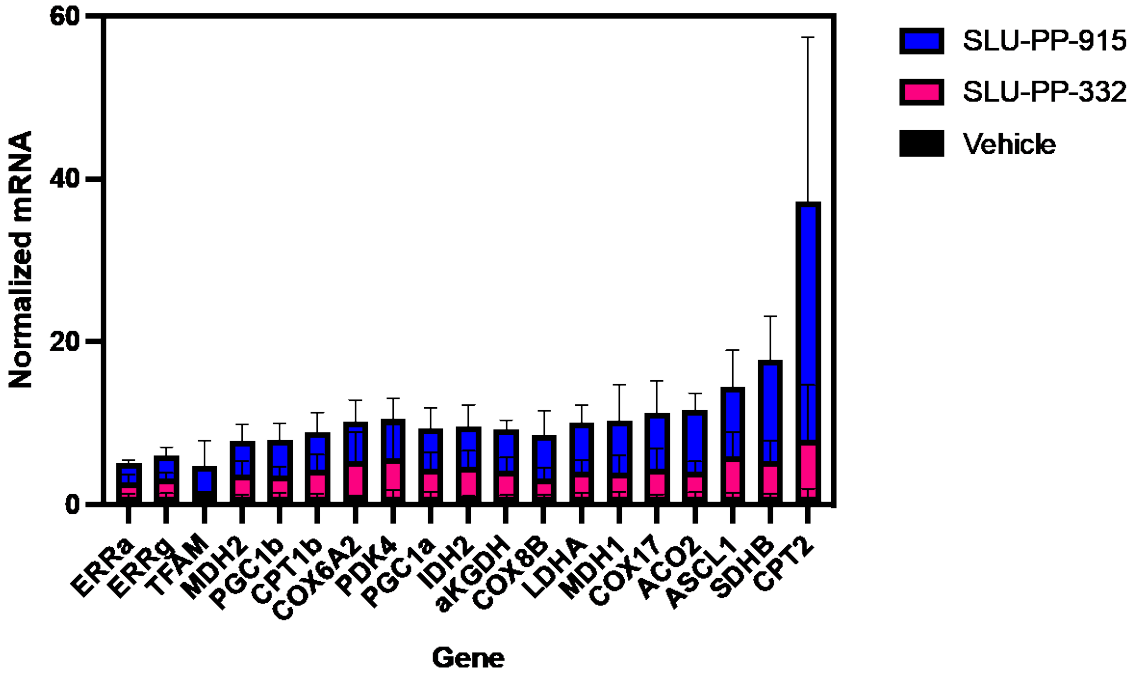

Figure 2.1: ERR agonists regulate gene expression of target genes *in vivo.* Wild type mice were treated for seven days with SLU-PP-332 or SLU-PP-915 at 50 mg/kg and 25 mg/kg, respectively, b.i.d. expression of ERR target genes was examined via qPCR.

2.4.2 Cytotoxicity of ERR agonists is assessed to gauge proper *in vitro* dosing

Prior to assessing the pharmacological effects of ERR agonists, we assessed their cytotoxicity in neonatal rat ventricular myocytes (NRVMs). NRVMs are a primary cell model commonly used to study cardiomyocyte metabolism and function. Although both *in vivo* and *in vitro* approaches are necessary, using *in vitro* models has advantages to *in vivo* studies such as more control over environmental factors, more flexibility for studying molecular mechanisms of interest, lower experimental cost to study biological effects. The disadvantages of NRVMs are they are not easily transfectable, do not divide like immortalized cells so require sacrificing animals for each assay, and do not fully recapitulate the microenvironment of the intact heart.

NRVMs are responsive to ERR ligands, as outlined below, and are valuable as a model system for addressing the therapeutic potential of ERRs in treatment of heart failure. Since NRVMs are fetal cardiomyocytes, their primary source of ATP production is via glycolysis, whereas adult cardiomyocytes rely on oxidative metabolism. In the failing heart there is a shift from oxidative metabolism to glycolytic; therefore, use of NRVMs allows investigation of this altered metabolic state.

Doxorubicin was used as a positive control for cell death and exhibited toxicity at all doses examined. At the highest dose of 10 μM, this compound induced approximately 75% cell death (**Figure 2.2A**). Cytotoxicity of SLU-PP-915 and SLU-PP-332 was assessed over a wide range of concentrations. SLU-PP-915 showed some toxicity at higher doses and we observed 25% cell death at 10 μM (**Figure 2.2B**). Lower concentrations were less toxic, which indicated that this compound could be used in functional studies. SLU-PP-332 displayed no cytotoxicity at doses up to 10 μM (**Figure 2.2C**).

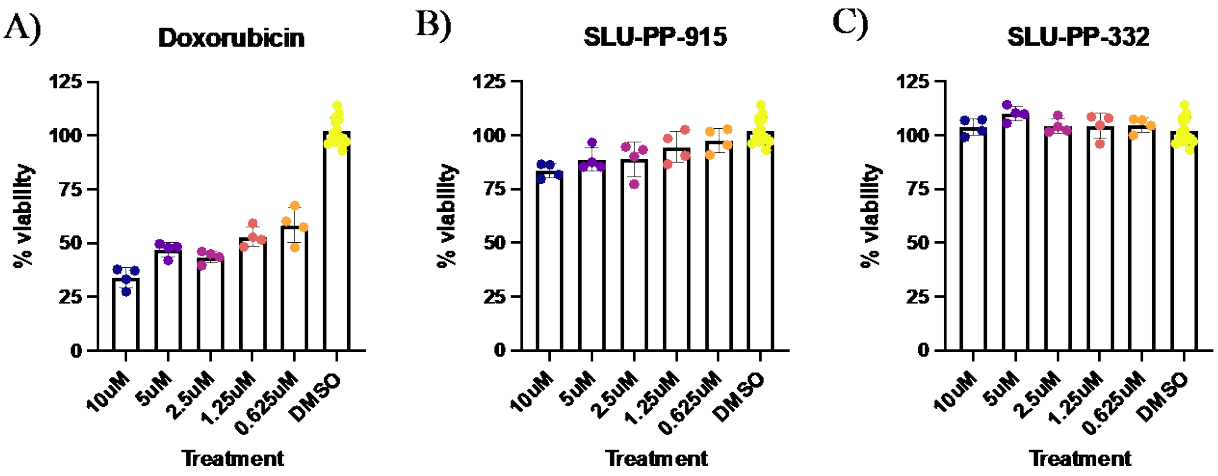

Figure 2.2: Cytotoxicity of ERR agonists was measured in NRVMs in a dose response manner. This assay helped determine proper *in vitro* dosing. A) Doxorubicin is a positive control and B-C) ERR agonists of interest. SLU-PP-915 shows toxicity at highest doses, so treatment was reduced to 2.5 μM for the remainder of experiments.

2.4.3 ERR Agonists Induce Expression of ERR Target Genes and Electron Transport Chain Proteins

After validating target gene expression *in vivo*, I examined gene expression changes in our NRVM primary model. After treating the cardiomyocytes for 72 h with SLU-PP-915 (5 μM), I ran a qRTPCR array plate to measure expression of cardiovascular related genes (**Figure 2.3**). The 72 h time point was chosen based on time course analysis studies looking at 24, 48, and 72 h of treatment (data not shown). Although target genes were modulated at each time point, 72 h was the most robust and consistent time point. The most upregulated transcript of the array was *Adra1b*, adrenergic receptor alpha 1B, which may be helpful in alleviating the toxic effects of hyperactivated adrenergic beta receptors in heart failure.[12] Pathological cardiac remodeling includes an increase of extracellular matrix fibrosis, specifically through increased deposition of

collagen fibers. Interestingly, after 72 h of treatment, SLU-PP-915 decreased both *Col3a1* and *Col11a1*, suggesting a potential anti-fibrotic effect.[13,14] This is consistent with previous studies in the lab that found a decrease in fibrosis of the hearts in an *in vivo* TAC model. *Hmgcr*, which encodes 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), the rate limiting enzyme in cholesterol biosynthesis, was also decreased. A common treatment for atherosclerotic cardiovascular disease, statins, target HMGCR through blocking the active site and suppressing the cholesterol synthetic pathway.[15] Upon further investigation, protein levels of HMGCR in cardiomyocytes were extremely low and almost undetectable with the antibody used (data not shown). *Hmgcr* is highly expressed in the liver, but since its expression is so low in the heart, this was not investigated further.

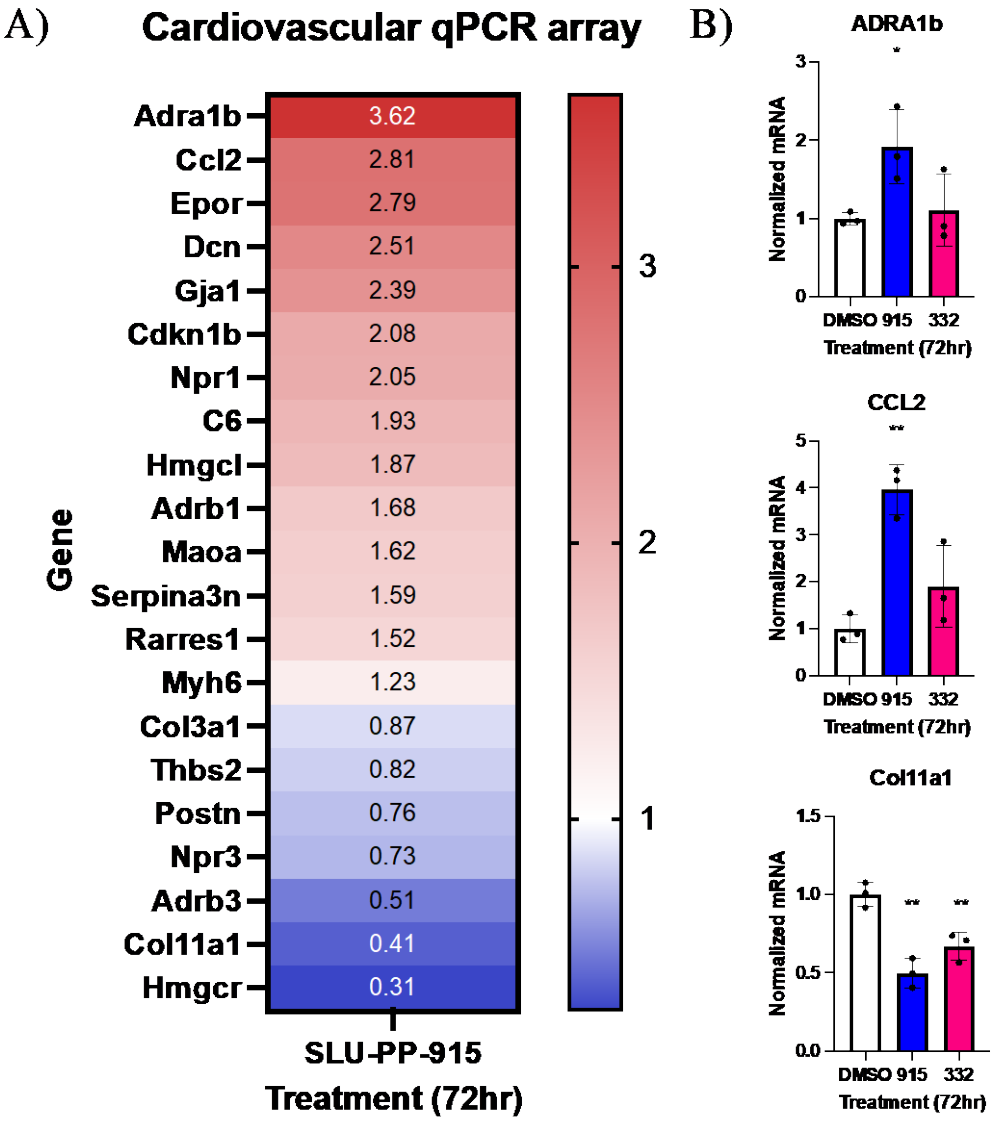

Figure 2.3: Genes involved with cardiovascular function are differentially expressed in NRVMs treated with ERR agonist. A) NRVM were treated for 72 h with SLU-PP-915 (5 μM), then ran on a cardiovascular qPCR array plate. B) Several genes were validated further with both compounds using qPCR from the array plate in A).

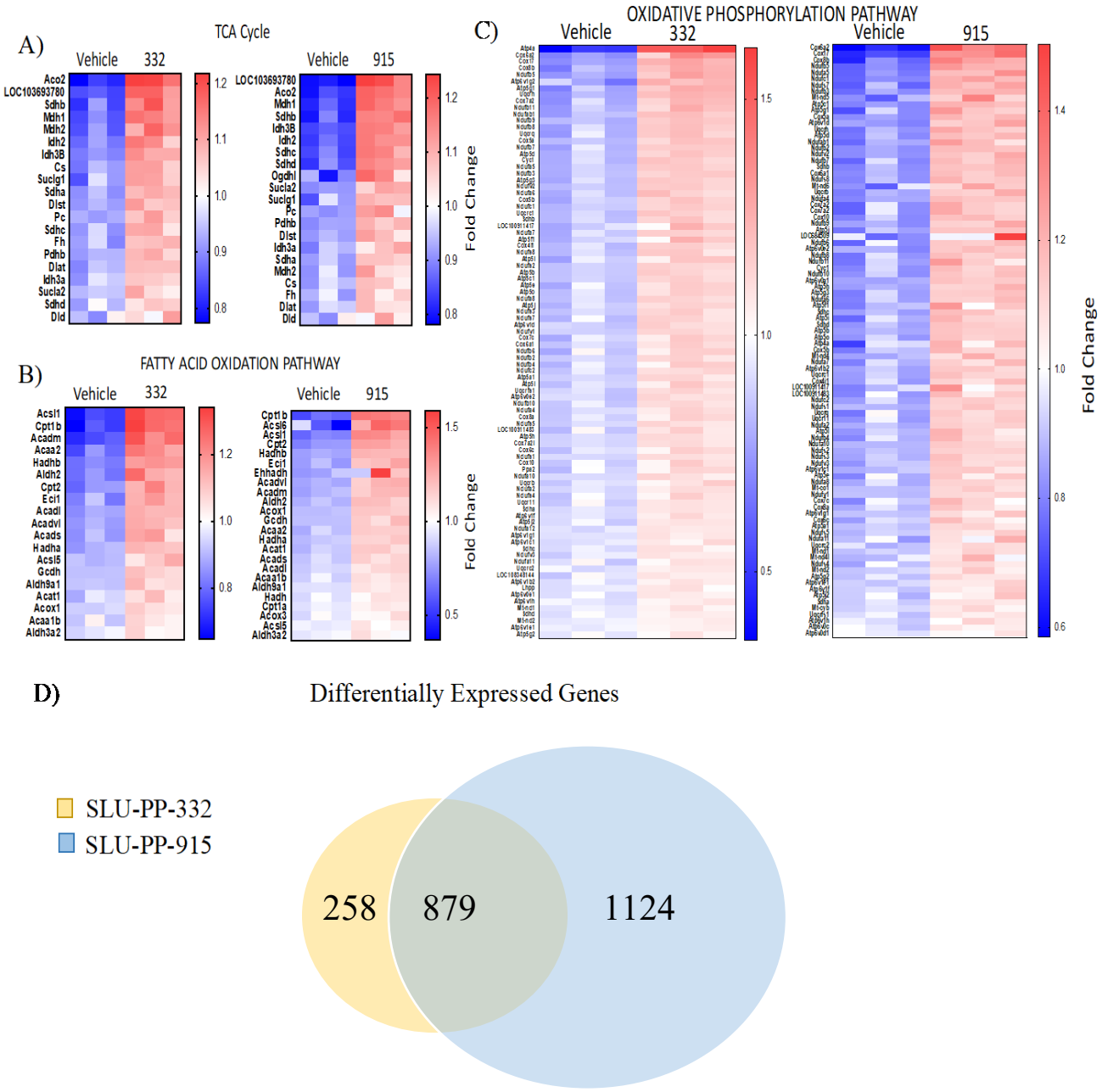

Figure 2.4: Transcriptomic data reveal pathway upregulation in NRVMs treated with SLU-PP-915 and SLU-PP-332. RNA-seq of NRVMs treated for 72 h with SLU-PP-332 or SLU-PP-915 (10 μM) significantly upregulate the A) TCA cycle pathway, B) Fatty Acid Oxidation Pathway, and C) Oxidative phosphorylation pathway. D) Venn diagram of differentially expressed genes comparing SLU-PP-332 and SLU-PP-915.

RNA-sequencing was also conducted to analyze gene expression changes in the NRVMs after 72 h treatment with both compounds (**Figure 2.4A-C**). We found broadly increased expression of pathways of genes in TCA, FAO, and OXPHOS pathways. The autophagy pathway was identified as differentially expressed in our dataset, which is novel for ERR and will be evaluated in Chapter 3 of this thesis. In this dataset, we found differential expression of 1,137 genes for SLU-PP-332 and 2,123 genes for SLU-PP-915 and both agonists shared a high degree of overlap in genes differentially expressed (**Figure 2.4D)**. Since SLU-PP-332 and SLU-PP-915 have unique structural scaffolds, but elicit highly similar gene expression response, we can confidently conclude that the responses are mediated by ERR.

Herein, we have confirmed transcriptional regulation of both known ERR target genes and other genes that are dysregulated in heart failure by novel ERR agonists. As indicated in Chapter 1, ERR knockout and overexpression studies have concluded contradictory results with regard to ERRs beneficial therapeutic utility in cardiovascular disease. ERRα/γ cardiac double knockout mice die prematurely due to heart failure phenotype, which points to the necessity of ERRs in physiological cardiac function.[16] However, ERRγ cardiac overexpression also results in a heart failure phenotype.[17] Although overexpression studies have their advantages, their clinical relevance may not be realistic considering the overexpression of ERRγ in the heart was >50-fold increase in the transgenic lines of the mentioned model.[17] This disadvantageous response to overexpression is also seen with overexpression of PGC1α leading to cardiomyopathy due to mitochondrial over proliferation.[18] With our ERR agonists we see modest activation of ERR and

mitochondrial respiration genes, which may provide a therapeutic window to avoid side effects seen in overexpression models. Future experiments will be required to determine a maximal therapeutic response with minimal side effects, using the *in vivo* TAC model studies done by the Burris lab as a starting point. With evidence for gene expression changes in these pathways, the next step was to study functional implications of these compounds.

After observing broad upregulation of OXPHOS genes with the ERR agonist treatment, I confirmed that this resulted in increased OXPHOS protein abundance in NRVMs (**Figure 2.5A**). Both compounds significantly increased the abundance of proteins from complex I-V in total cell lysates from NRVMs (**Figure 2.5B**). Increases in the range of ~1.3-1.5-fold were observed, which is similar to the magnitude of increase observed in gene expression from the RNA-seq analysis. These findings suggest that ERR agonism is sufficient to enhance the expression of several components of the OXPHOS complexes in cultured NRVMs.

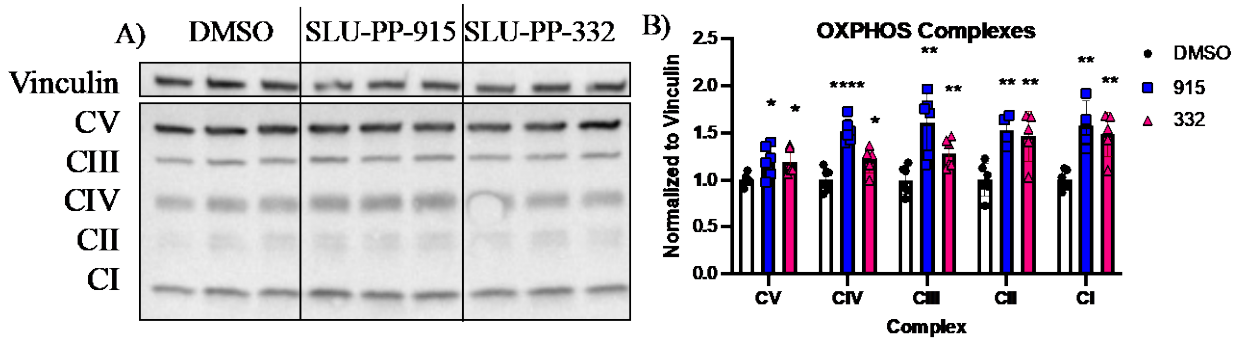

Figure 2.5: Electron transport chain proteins expression are increased with treatment of ERR agonist in NRVMs. A) Western blot of OXPHOS complex I-V proteins using an OXPHOS antibody cocktail, with treatment of SLU-PP-332 (10 μM) and SLU-PP-915 (5 μM). B) Quantification of Western blots looking at OXPHOS complex I-V.

2.4.4 Mitochondrial Content and Activity Increases with Treatment of ERR agonists

Given the alterations in mitochondrial gene and protein expression, we began to examine the effects of ERR agonism on mitochondrial function by assessing MitoTracker red staining via flow cytometry. MitoTracker red is an indicator of mitochondrial membrane potential and the relative activity of the mitochondria.[19] Mitotracker red staining was significantly increased by both SLU-PP-332 and SLU-PP-915 after 24 h treatment (**Figure 2.6A-B**). An increase in mitochondrial membrane potential reflects an enhanced respiratory capacity and ATP synthesis, which are decreased in the failing heart.[20] Our data illustrate that ERR agonists increase mRNA and protein expression of OXPHOS machinery coupled with the increase in mitochondrial membrane potential and indicate that the compounds effectively increase mitochondrial activity.

Next, we analyzed oxygen consumption rates of mouse cardiomyocytes using Seahorse XF96 following treatment with ERR agonists. This experiment relies on the principle that oxidative phosphorylation consumes oxygen to generate ATP. Using either palmitate or pyruvate as substrates, we observe a significant increase in maximum oxygen consumption rates and mitochondrial respiratory capacity after treatment with SLU-PP-915 for 24 h (**Figure 2.6C-E**). Both substrates were evaluated to assess if treatment shifted the cells substrate preference (pyruvate – pyruvate oxidation vs. palmitate – fatty acid oxidation). However, we found that respiration was increased in the presence of either pyruvate or palmitate. These results are consistent with our results indicating an increase in OXPHOS gene/protein expression. Of note,

there are no significant differences on the other arms of the Seahorse trace: basal respiration before oligomycin addition and ATP-linked respiration after oligomycin respiration.

Measuring fatty acid oxidation rates was an important next step in understanding the therapeutic role modulating ERR could play in reversing metabolic dysfunction in heart failure since FAO is decreased in the failing heart. WY14643 is a PPARα agonist that was used as a positive control due to its known effect of significantly increasing FAO rates.[21] Etomoxir was used as a negative control because it significantly decreases FAO by irreversibly inhibiting CPT1, the rate limiting step of this pathway.[22] Fatty acid oxidation rates were significantly increased for both SLU-PP-332 and SLU-PP-915 after 72 h treatment in NRVMs (**Figure 2.6F**). Rates of palmitate oxidation were calculated using Equation 1, where Δ is the difference between the sample DPM and the mean blank and mRDA is the average of the radioactive blank. We observe approximately a 10% increase in FAO rates with our ERR agonists compared to a 30% increase with our positive control.

Equation 1: $$\boldsymbol{Results}\left(\frac{nmol\,^3H\text{-}FA}{hr*mg\ protein}\right) = \frac{\Delta}{mRDA} \times \frac{1}{ug\ protein}$$

This modest but significant increase is an important result because increasing FAO too much could lead to detrimental effects due to excessive ROS production. PPAR agonists have been hypothesized to be a therapeutic approach to treating heart failure due to their known transcriptional regulation of fatty acid oxidation.[23] However, in mice with PPARα overexpression or use of highly potent PPARα agonists leads to cardiac dysfunction.[24,25,26] The authors concluded

the cardiac dysfunction is due to the 2.5-fold activation of PPARα, leading to increased fatty acid oxidation levels that surpass the cardiomyocytes capacity, leading to reduced cardiac efficiency.[26] We demonstrate that ERR regulates fatty acid oxidation to a lesser extent than PPAR activation, potentially avoiding the detrimental cardiac effects observed in the previously mentioned studies.

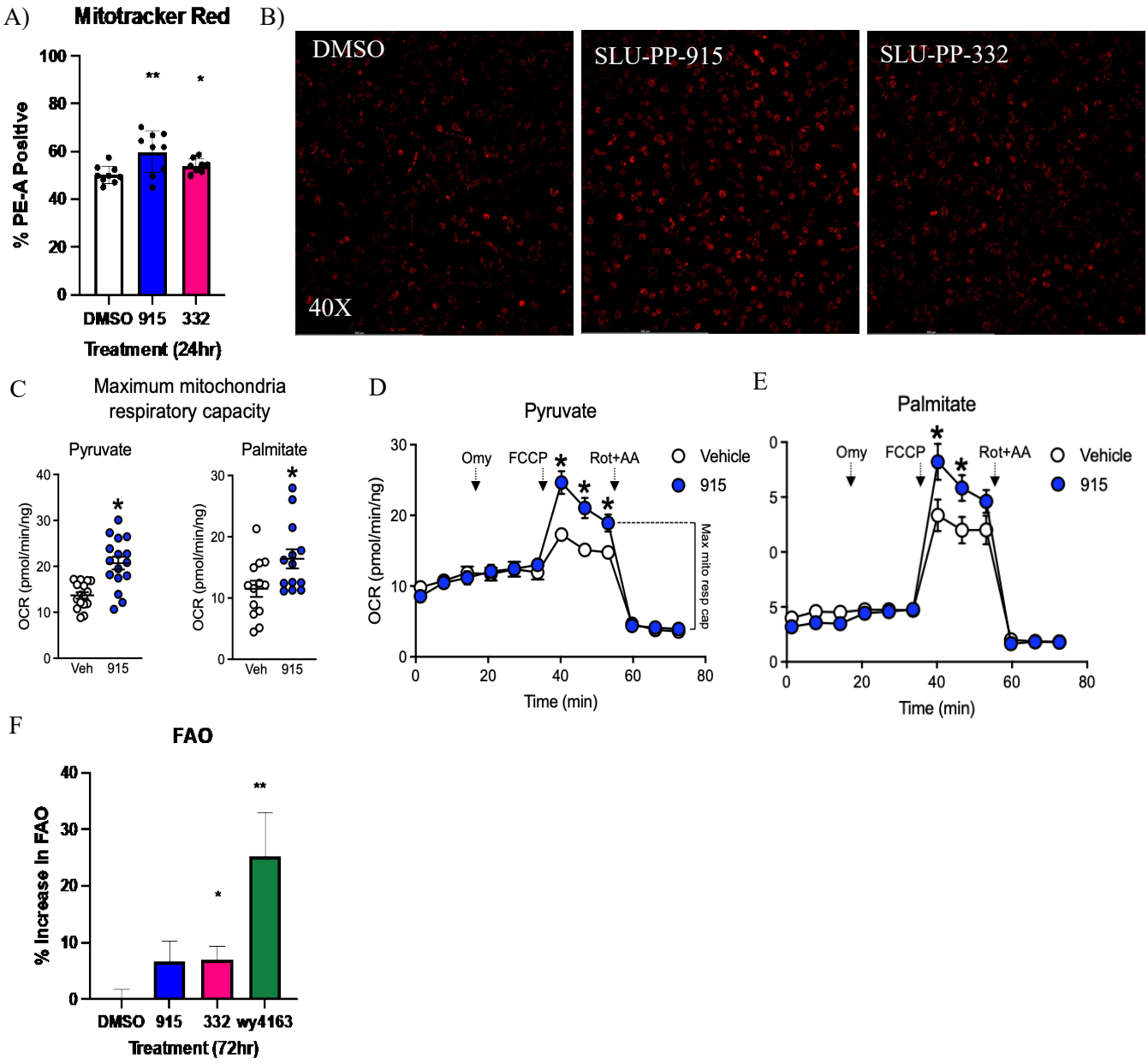

Figure 2.6: Mitochondrial activity increase with treatment of ERR agonists. A) Mitotracker red staining in NRVMs evaluated by flow cytometry. B) Representative imaging of Mitotracker red staining of each treatment condition in NRVMs. C-E) Oxygen consumption rate of mouse cardiomyocytes was measured via Seahorse XF analyzer after 24 h treatment with SLU-PP-915 (10 μM). Treatment with SLU-PP-915 led to a significant increase in maximal mitochondrial respiratory capacity compared to vehicle using either B) pyruvate or C) palmitate. F) Fatty acid oxidation rates were measured in 72 h treated NRVMs by adding radioactive palmitate for 2 h and scintillation counting to quantify tritiated water production.

To assess changes in mitochondrial content and TCA cycle activity, a citrate synthase activity assay was conducted in C_2C_{12} cells were treated with SLU-PP-915. Citrate synthase catalyzes a rate limiting step in the TCA cycle where acetyl-coA is condensed with oxaloacetate to form citrate.[27] This absorbance-based assay measures citrate synthase activity in a sample, which correlates to mitochondrial content. After 24 h SLU-PP-915 treatment of C_2C_{12} cells, there was a significant increase in citrate synthase activity (approximately 6%) (Figure 2.7A). To further confirm changes in mitochondrial content, C_2C_{12} cells were treated with SLU-PP-915 and mtDNA quantified by qPCR. Since mitochondria have their own DNA, mtDNA content can be determined by normalizing the CT values for mitochondrial DNA by genomic DNA. We found that DNA for six of the seven mitochondrially-encoded genes assessed was significantly increased after 48 h treatment with SLU-PP-915 (5 μM) (Figure 2.7B). This indicates that SLU-PP-915 significantly increases the mitochondrial content in C_2C_{12} cells.

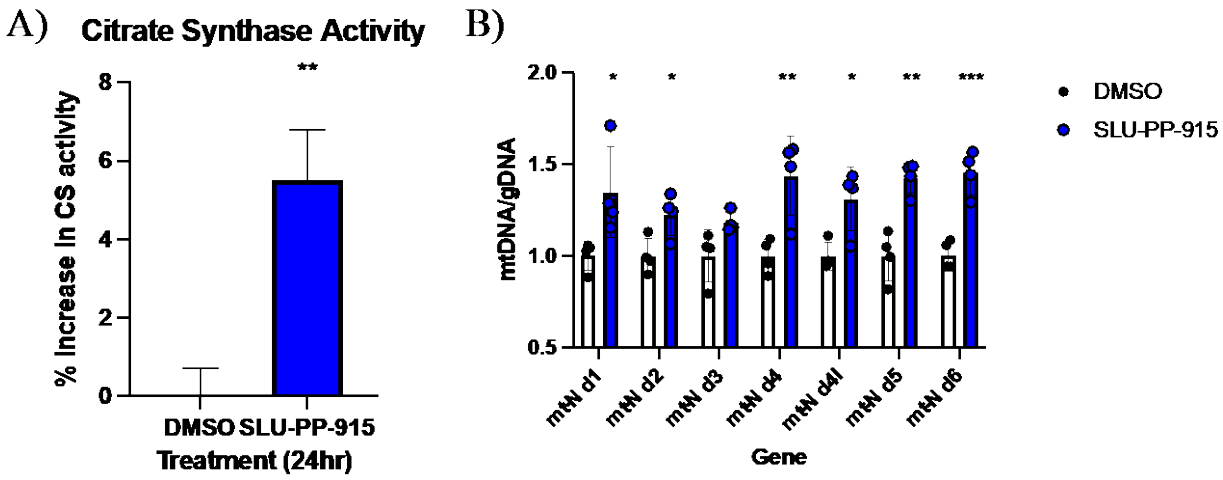

Figure 2.7: Mitochondrial content increases with ERR agonists. A) Citrate synthase activity rates increase after 24 h treatment with SLU-PP-915 (5 μM), indicating an increase in mitochondrial content. B) C_2C_{12} cells treated for 48 h with SLU-PP-915 (5 μM) have significant increase in DNA for six of seven mitochondrially-encoded genes, indicating an increase in mitochondrial content.

In models of heart failure, reduced mitochondrial biogenesis and activity has been observed, and correcting these processes has been a proposed therapeutic intervention. mtDNA copy number is decreased in heart failure, and when mtDNA content is decreased mtDNA-encoded protein expression are also decreased.[28] Here we show an increase in mitochondrial content and activity with ERR agonist treatment, highlighting the therapeutic potential for normalizing energy homeostasis through improving functionality of mitochondria in the disease context. Impaired mitochondrial capacity and function has been reported in patients with both HFrEF and HFpEF, so targeting this disease aspect could be beneficial for both patient populations.[29] As mentioned before, no successful therapeutic interventions exist for HFpEF, so future investigations on this specific type of heart failure are necessary.

2.5 Conclusions

We hypothesized that pharmacologically targeting ERRα/γ would reverse an array of metabolic pathways that are pathologically inhibited in the failing heart. We found that both *in vivo* and *in vitro* cardiac models show a change in gene expression profiles upon treatment with ERR agonists SLU-PP-332 and SLU-PP-915. Specifically, in pathways such as TCA, FAO, and OXPHOS we see global pathway upregulation at the transcriptional level upon treatment. Protein expression of OXPHOS related proteins, mitochondrial content, and citrate synthase activity were also increased with ERR agonist treatment. We also detected increased rates of fatty acid oxidation maximal mitochondrial respiration in cardiomyocytes treated with ERR agonists. Our results validate the role of ERR in energy and metabolic regulation: pathways which are dysregulated in

heart failure. The functional studies performed further elucidate the mechanism of ERR regulation in these pathways and open the door to continuing to unveil the therapeutic utility of ERR activation for heart failure treatment.

2.6 Methods

2.6.1 NRVM Isolation

To isolate NRVMS, postnatal day 0-3 rat pups were sacrificed and their hearts dissected to separate atria from ventricular tissue. The hearts were then rinsed in HBSS (ThermoFisher Scientific, 14025092) and minced into quarters before placing in trypsin-EDTA 0.05% (ThermoFisher Scientific, 25300120) at 4^{o}C overnight. The next morning the tissue underwent several rounds of digestion with collagenase type II (ThermoFisher Scientific, 17101015) in HBSS. After little to no tissue matter was left to digest, the cells were pre-plated for 90 minutes at 37^{o}C with 5% CO_2 incubation to remove fibroblasts, macrophages, and other non-parenchymal cells. The supernatant from the pre-plating was collected, cells spun down and counted before seeding in Gibco DMEM (ThermoFisher Scientific, 10566024) with 10% FBS, 1% pen/strep, and 100uM BRDU (Sigma-Aldrich, B5002). The media was changed to DMEM with .1% ITS (PeproTech, 00102) after 48 h incubation and the cardiomyocytes are used for experimentation.

2.6.2 Gene Expression Studies

Compounds for experimentation were prepared at 10 mM in DMSO, which was then diluted to 10 μM, 5 μM, or 2.5 μM for *in vitro* assays. Stock solutions were flushed with nitrogen, aliquoted,

and stored at -20^{O}C to prevent degradation. RNA was extracted from NRVMs using RNeasy Mini Kit (Qiagen, 74106) per the manufacturers protocol. To obtain cDNA from RNA, reverse transcriptase was performed using iScript Reverse Transcription Supermix (Quanta, 95047). For qPCR analysis, Sybr Green Supermix (Thermo Fisher, 4309155) was used and analyzed in Quant Studio 5 Real-Time PCR System. Expression levels were normalized by PPIB control and ΔΔCt-method for calculation, and each experiment consisted of n=3 biological replicates and n=2 technical replicates.

2.6.3 Protein Expression Studies

To extract protein from NRVMs, the cells were lysed with RIPA buffer (VWR, 97063-270) supplemented with protease inhibitor cocktail (Sigma-Aldrich, 05056489001). Protein concentration was determined with Pierce BCA assay kit (ThermoFisher Scientific, 23227). Samples were mixed with 95% Tru-Page loading buffer (Sigma-Aldrich, PCG3009) plus 5% 1 mM DTT before boiling for 5 min at 95 ^{O}C. Samples were loaded on 4-20% gels with TGS buffer (Fisher Scientific BP1341-1) and transferred to PVDF membrane (Bio-Rad, 1704156EDU) using the BioRad trans-blot turbo transfer system. The membranes were incubated in 5% nonfat milk in TBST for 1 h at room temperature. Primary antibody was diluted 1:1000 and incubations were overnight at 4^{O}C. The primary antibody used to assess OXPHOS proteins was OxPhos Rodent WB Antibody Cocktail (ThermoFisher Scientific, 458099). Secondary antibodies were diluted 1:3000 and incubated for 1 h at room temperature (Cell Signaling Technology, 7076P2). The western blots were imaged iBright imaging system and bands quantified using their software.

2.6.4 Fatty Acid Oxidation Assay

NRVMs were plated in 12-well plates and treated for 72 h with compounds. The ion exchange resin columns were prepared by sealing the end of a 9-inch Pasteur pipet with a Bunsen burner. Fiberglass (VWR 32848-003) was inserted into the pipette and pressed towards the bottom until approximately 1 cm thick. Ion exchange resin (19.9 g) was weighed (DOWEX 1x2-400 Sigma, 217395) and stirred for 10 min with 54 mL of Milli-Q water. Resin (2.4 mL) was added to the sealed pipettes, ensuring there were no bubbles remaining in the pipette. The columns were stored at 4 ^{o}C overnight and used for experimentation 1-3 days after preparation. Palmitate-BSA (Perkin Elmer, NET-043, Sigma, A7511) was diluted from stock to 125 μM with PBS + Ca-Mg (Sigma, D1283) and 1 mM carnitine (Sigma C0158) was added. Radioactive palmitate is added to the cells for 2 h. Before the end of the 2 h incubation, pipette tips are broken off and allowed to drain. The supernatant from the cells was mixed with 10% trichloroacetic acid and centrifuged at 3300 rpm for 10 min at 4 ^{o}C. The supernatant was neutralized with 6 M NaOH and the total volume applied to the resin filled pipettes. A scintillation vial was used to collect the flow through from the column, which was washed with 1.7 mL of distilled water. Radioactivity of the eluent mixed with 5 mL of scintillation fluid was measured by scintillation counting. Radioactive counts were normalized to protein content of each well quantified by using a Pierce BCA assay kit.

2.6.5 Flow Cytometry with Mitotracker Staining

NRVMs were treated with SLU-PP-332 (5 μM), SLU-PP-915 (2.5 μM), or DMSO control 24 h prior to analysis. The cells were lifted with Trypsin-EDTA and pelleted at 1000 rpm for 5 min. The pellet was resuspended in MitoTracker Red (400 nM) in PBS for 1 h. The cells were pelleted

and washed with PBS twice before putting the suspended cells in FACS tubes. The suspended cells were vortexed to obtain single cell suspension and run on the BD Accuri flow cytometer. The results were gated for live positively stained cells and treatment groups compared to DMSO control.

2.6.6 Mitochondrial DNA quantification Assay

C_2C_{12} cells were seeded at 50,000 cells/well in a 12-well plate. The next day the cells were treated for 48 h with SLU-PP-915 (5 μM) and the media with drug was replaced every 24 h. DNA was extracted from the cell using DNeasy blood and tissue kit (Qiagen, 69504), loaded on a qPCR plate, and nuclear and mtDNA content measured by using primers specific for mitochondrial- (mt-Nd1-6) or nuclear-encoded NDUFV1 genes. For qPCR analysis, Sybr Green Supermix (Qiagen, 330523) was used and analyzed in Quant Studio 5 Real-Time PCR System. The ddCt-method for calculation was used to quantify the ratio of mtDNA to nuclear DNA and each experiment consisted of n=4 biological replicates and n=2 technical replicates.

2.6.7 Citrate Synthase Assay

C_2C_{12} cells were seeded at 200,000 cells /well in a 6-well plate. The next day the cells were treated for 24 h with SLU-PP-915 (5 μM) or SLU-PP-332 (10 μM). The cells were collected and prepared for citrate synthase activity per the manufacturer's protocol (Abcam, ab239712). Absorbance is read with a plate reader.

2.7 References

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Chapter 3: ERR Agonists Increase Autophagy

3.1 Preface

Parts of this chapter are modified from a paper published on the bioRxiv preprint server (https://doi.org/10.1101/2023.03.02.530836). The work outlined in this chapter was done in collaboration by myself, Cyrielle Billon, Matthew Hayes, Aurore Valfort, Weiyi Xu, Thomas Burris, and Lilei Zhang. In this chapter, I am the first author, responsible for a majority of manuscript writing and experimental assays including designing, executing, and analyzing data of experiments for i) qRTPCR gene expression assays, ii) western blotting assays, iii) luciferase reporter assays, iv) nuclear fractionation, and v) immunofluorescence studies. TB and CB are both PIs on this paper and responsible for designing and coordinating research in this collaboration. MH assisted in data analysis of the NRVM RNA-sequencing data and motif analysis. AV constructed the TFEB deletion construct. Specifically, data shown in this chapter that were performed by others include i) NRVM RNA-sequencing.

3.2 Abstract

Proper control of autophagic rates is essential for physiological cardiac function and prior work has demonstrated that autophagy is decreased in the failing heart. By pharmacologically targeting the nuclear hormone receptor estrogen related receptor α and γ (ERRα/γ), we increased autophagy flux in cardiomyocytes suggesting the utility of activating ERRs to enhance autophagy

in failing heart. ERRα and γ knockout mice exhibit heart failure, illustrating their critical role in cardiac function. Reduced ERR expression is also implicated in heart failure in humans. Thus, we focused on pharmacological targeting of these receptors to treat heart failure. Neonatal rat ventricular myocytes (NRVMs) treated with ERR agonists show an increase in autophagy flux as measured by markers such as LC3 and p62 protein. RNA-seq data from NRVMs treated with ERR agonist demonstrated enhanced expression of genes within the autophagy pathway. ERR was found to directly increase transcription factor EB (TFEB), a master regulator of the autophagy-lysosome pathway. Our data suggest a novel role for ERR activation in enhancing the activity of the autophagy pathway, suggesting that ERR can be targeted to remedy autophagic deficits that occur in heart failure as well as other diseases.

3.3 Introduction

Autophagy is a conserved degradative pathway in the cell that allows removal and/or recycling of old or abnormal proteins or organelles. This process allows the cell to maintain cellular homeostasis and when autophagy is dysregulated this can play a role in the pathophysiology of many diseases. Autophagy gradually decreases with aging and has been postulated to play a pivotal role in many aging-related diseases including cardiovascular, musculoskeletal, neurodegenerative, and metabolic disorders.[1]

The three main types of autophagy are [1] chaperone mediated autophagy (CMA), [2] microautophagy, and [3] macroautophagy. CMA is a selective type of autophagy where substrates are sequestered by chaperones, such as heat shock proteins (HSP), and traffic to the lysosome for

degradation.[2] Microautophagy is a non-selective form of autophagy where the substrate is captured directly by the lysosome with the main purpose of maintaining organelle size, membrane homeostasis, and cell survival.[3] Macroautophagy is characterized by phagophore formation that allows capture of substrate followed by autophagosome and autolysosome formation for targeted degradation of substrates. Macroautophagy is induced by various stimuli (starvation, oxidative stress, or hypoxia) that deactivate the mechanistic target of rapamycin (mTOR) and cause its dissociation from the ULK1 complex, thus activating ULK1.[4,5] Subsequently, the ULK1 complex phosphorylates PI3KC3 complex 1, stimulating PI3P production and the formation of a structure on the ER called the omegasome.[6] At the site of the omegasome, phagophore formation begins by recruitment of WIPI, ATGs, and LC3II, which form and expand the phagophore membrane.[7] As the phagophore expands, substrates are sequestered to be engulfed by the membrane. LC3 is involved in sequestration of labeled substrates containing LC3-interacting regions (LIRs). Many proteins are tagged with ubiquitin for degradation and p62 (SQSTM1) helps recognize and bind these labeled substrates.[6,8] Once the substrate is sequestered, the autophagosome seals and matures, ready for fusion with the lysosome for degradation. Key players in facilitating fusion of the autophagosome to the lysosome are motor proteins (dynein, kinesin), tethering factors (RABs, HOPS), and fusion machinery (SNAREs).[9] Proteolytic hydrolases such as papain-like cysteine proteases, pepsin-related aspartyl proteases, and asparaginyl endopeptidases degrade the substrate.[10] The degraded material may be recycled to be used by the cell for a variety of purposes.

Mitophagy is a specific form of macroautophagy where mitochondria are targeted for elimination. Mitophagy occurs in a selective or non-selective manner in response to cellular conditions such as oxidative stress, starvation, hypoxia, and mitochondrial membrane potential

collapse.[11] Mitophagy can be induced in a Parkin-dependent or Parkin-independent manner. Parkin is recruited to the mitochondria subsequent to a damaging event, such as membrane depolarization, and promotes ubiquitination of mitochondrial proteins to be recognized by p62. Substrate recognition by LC3 and p62 recruit the mitochondria to the autophagosome.[12] Parkin-independent mitophagy inducers include BNIP3, NIX, FUNDC1, BCL2L13, and others.[13] The balance between mitophagy and mitochondrial biogenesis is crucial in supporting cellular energetics.

A master regulator of the autophagy-lysosome pathway is TFEB (transcription factor EB) that regulates the expression of an array of genes involved in autophagosome formation, fusion with the lysosome, and lysosomal biogenesis. This transcription factor is part of the microphthalmia/transcription faction E (MiT/TFE) basic helix-loop-helix-leucine-zipper (bHLH-Zip) family that regulates genes containing a CLEAR (Coordinated Lysosomal Expression and Regulation) motif.[14] Other related transcription factors in this family are TFE3 and TFEC.[10] TFEB is phosphorylated in its inactive state, but once activated the transcription factor is dephosphorylated and translocates to the nucleus to regulate transcription.[15] PGC1α, PPARα, calcineurin, and PKC are known activators of TFEB and mTORC1, AKT, and MAPK1 are inactivators.[16,17,18] Germline knockout of TFEB results in embryonic lethality.[19] Cell specific knockout of TFEB in both NRVMs and C_2C_{12} cells lead to reduced autophagic flux while overexpression enhances autophagic flux and lysosomal biogenesis (**Table 3.1**).[20,21,22] These studies highlight the importance of TFEB in autophagy homeostasis in heart and muscle.

Alteration in TFEB expression	Phenotype
Whole body TFEB KO	Embryonic lethal
NRVM TFEB KO	Reduced autophagy flux
NRVM TFEB overexpression	Enhances autophagy flux
Muscle TFEB KO	Reduced autophagy flux
Muscle TFEB overexpression	Enhances autophagy flux and lysosomal biogenesis

Table 3.1: Knockout and overexpression studies of TFEB in cardiac and muscle tissue highlight the important role it plays in the autophagy pathway.

Since autophagy is dysregulated in a variety of diseases, finding a therapeutic target to activate TFEB is of great interest. There are several compounds identified that activate TFEB either directly or indirectly. Compounds that directly target TFEB include resveratrol, curcumin analog C1, progestin and while Torin1 and rapamycin indirectly target the protein.[18] Some of these compounds have undergone preclinical or clinical evaluation for a variety of disease indications and have yielded promising results, but more specific and efficacious activators are needed to eliminate undesirable off target effects.

Cardiomyocytes rely on the autophagy-lysosome pathway to drive turnover of old or abnormal organelles and proteins allowing for maintenance of homeostasis. Cardiomyocytes are

mitochondria-rich cells due to the metabolic rates they must maintain to fuel continuous cardiac contraction and relaxation. Damaged mitochondria also release pro-apoptotic signals that cause programmed cell death, but autophagy can recycle these defective mitochondria to prevent apoptosis to maintain cellular lifespan.[23] In the failing heart, autophagy is decreased, in association with an increased rate of cardiomyocyte cell death. Currently, there are no treatments for heart failure that target cardiac autophagy. However, there is great interest in finding pharmacologic agents to increase cardiac autophagy that would be expected to decrease mitochondrial damage, oxidative stress, inflammation, and apoptosis that occurs in the failing heart.[24]

Many studies have been performed assessing the beneficial effect of TFEB in cardiovascular disease. Activation of TFEB may be cardioprotective since TFEB expression is decreased in mice with heart failure (via TAC) vs. normal mice.[23] Recent work showed increasing autophagy flux through increased expression of TFEB (by AAV transfection) was beneficial in reversing left ventricular remodeling in mice that had heart failure (via TAC and myocardial infarction) coupled to hemodynamic unloading through debanding.[25] Additionally, increased expression of TFEB in a BNIP3 adenoviral transduced model in neonatal rat cardiac myocytes was found to reverse the effect of BNIP3-induced cell death by increasing lysosomal degradation and enhancing the removal of depolarized mitochondria.[26] The role of TFEB in cardiac autophagy is further demonstrated by the observations that TFEB is decreased in patients with heart failure, protects against proteotoxicity, normalizes desmin location, reduce inflammatory cytokines, and diminishes myocardial dysfunction post injury.[27,20,28,29]

The role of ERRα in regulating autophagy has not been extensively examined and the effects of activating ERRα in cardiac autophagy has not been investigated at all to our knowledge. One study found that ERRα induced expression of autophagy-related (Atg) genes and knockout resulted in decreased phagosome maturation in the context of innate antimicrobial defense against mycobacterial infection in bone marrow-derived macrophages.[30] Another study found that ERRα null mice had significantly decreased AMPK, TFEB, and p62 levels compared to WT mice when studying the role of ERRα in intestinal homeostasis.[31] Of note, we are aware of no studies examining the effects of ERRβ and γ in autophagy. PGC1α is a known co-activator of ERRs, acting as a protein-ligand to change the activation domain of the receptor into an active conformation to enhance the expression of target genes.[32] PGC1α is also a known regulator of the autophagy pathway; one way PGC1α modulates autophagy is through activation of TFEB. Further investigation of the role of ERR in autophagy and the link with TFEB will address a gap in our knowledge of the regulation of cardiac autophagy.

As I studied the mechanistic and functional assays discussed in this chapter, I realized the limitations NRVMs have for studying autophagy. As mentioned previously, NRVMs are not easily transfectable. I attempted many transfections of these cells using several transfection reagents without success. This limitation prompted me to characterize another cell line to be used for experiments that NRVMs could not. The murine skeletal muscle cell line, C_2C_{12}, was chosen due to its ease of transfection, its endogenous expression of ERRs, and the relevance of ERR function to skeletal muscle. Given that both NRVMs and C_2C_{12} cells are muscle cells we believed that there would be similarities in ERR/TFEB/autophagy function even though they are distinct classes of

muscle cells. C_2C_{12} cells can differentiate from myoblasts to myotubes and during this differentiation process they switch from glycolytic to oxidative, much like NRVMs.[33] Although C_2C_{12} cells have their own limitation in the context of studying heart failure, they allowed for addressing the mechanistic studies I performed to dissect the ERR-autophagy axis.

3.4 Results and Discussion

3.4.1 ERR Agonists Induce Expression of Autophagy Related Genes

To examine gene expression changes in autophagy related genes, NRVMs were treated with SLU-PP-915 (5 μM) for 72 h. I then isolated RNA and used a qRTPCR array to measure expression of autophagy related genes (**Figure 3.1**). With an impressive 5.7-fold increase in expression, *Irgm* was the most up-regulated gene in response to SLU-PP-915. Immunity related GTPase M (*Irgm*) is involved with organizing the core autophagy machinery by interacting with *Ulk1* and *Beclin1* to promote assembly.[34] Additionally, expression of *p62* was upregulated 4-fold by SLU-PP-915. As previously mentioned, *p62* is involved in facilitating the degradation of ubiquitinated proteins through autophagy.[8] Of note, many ATG genes that play a role in autophagosome formation and sealing were up-regulated by SLU-PP-915, including *Atg12, -4c, -16L1, -9a, -101,* and *-3*.[35] The gene with the largest decrease in expression after SLU-PP-915 treatment in the autophagy array plate was *Snca*, also known as α-synuclein. α-synuclein is a known contributor to neurodegenerative diseases such as Parkinson's disease, but the role in the heart is unknown.[36] For the purpose of this work I did not delve deeper into α-synuclein; however, this has opened a door for future direction studies for our ERR agonists in neurodegenerative diseases.

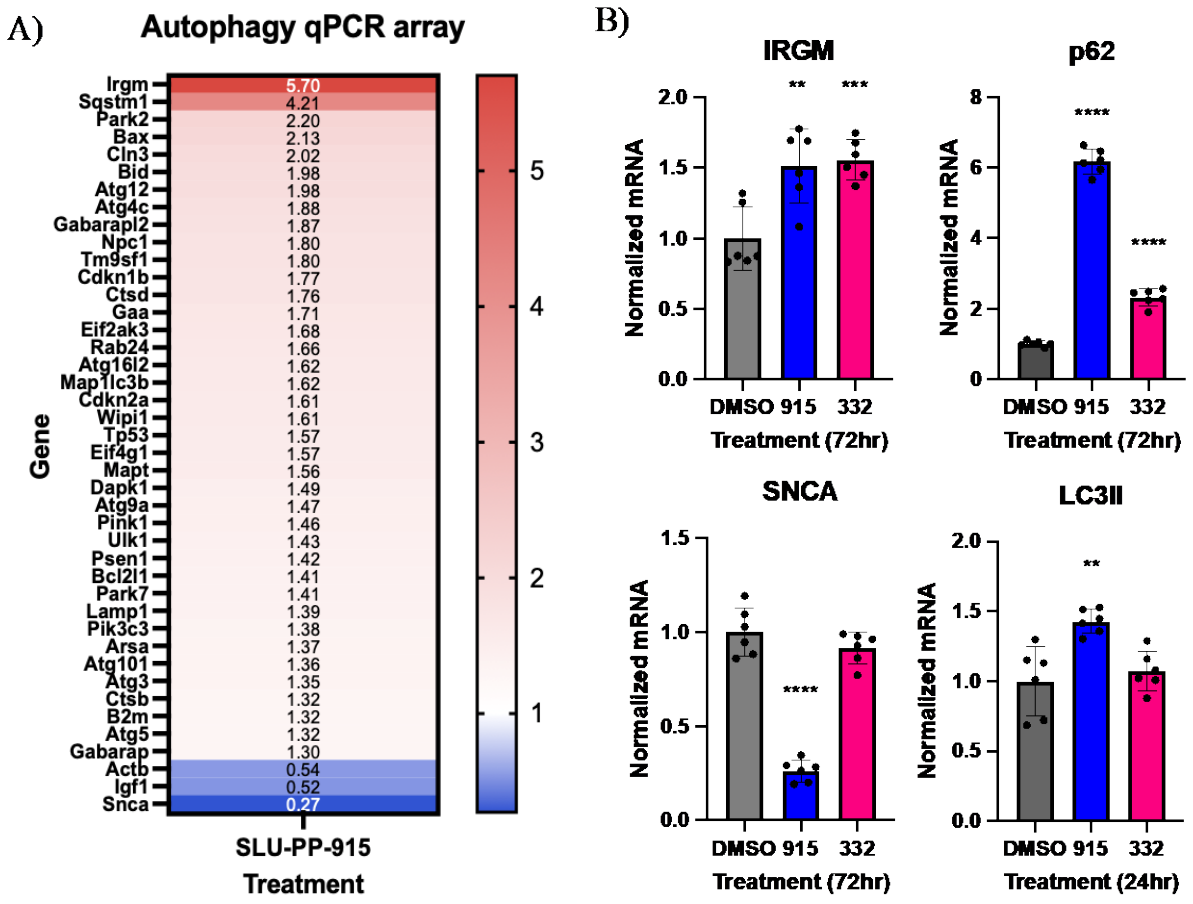

Figure 3.1: Genes involved with autophagy pathway are differentially expressed in NRVMs treated with ERR agonist. A) NRVM were treated for 72 h with SLU-PP-915 (5 μM), then gene expression was measured by using an autophagy qPCR array. B) Several genes from the array in (A) were validated further with both ERR agonists using qRTPCR.

To understand how SLU-PP-915 induces autophagy, I turned to the immortalized murine myoblast (C_2C_{12}) cells. Based on a time course of SLU-PP-915 response in C_2C_{12} cells, an acute treatment of 3 h and a sustained treatment of 48 h was chosen (**Figure 3.2A-B**). At the 3 h time point, expression *Atg12 and Atg10* was most highly induced in response to SLU-PP-915. Interestingly, expression of *Mtor* (mechanistic target of rapamycin) was decreased by almost half compared to DMSO control. mTOR is a negative regulator of autophagy that in nutrient rich condition inhibits recruitment of autophagy initiation complexes and nuclear translocation of

TFEB.[37] In response to cellular stress, such as starvation, mTOR becomes inactivated causing autophagy to be derepressed. After 48 h of stimulation with ERR agonist, there were several genes with increased expression involved in autophagy initiation, formation, and expansion (*Atgs, p62, Lc3, Becn1, Ulk, etc.).* Comparison of the three datasets revealed 8 overlapping genes regulated in all experiments (**Figure 3.2C**). One example of these genes was *Akt1*, which is a ser/thr kinase that was decreased by SLU-PP-915 in each dataset. AKT1 inhibits TFEB nuclear translocation, so decreasing the expression of AKT1 may promote TFEB translocation. Another important observation was at the 48 h and 72 h treatment for C_2C_{12} and NRVMs respectively, there was a large overlap in genes regulated by SLU-PP-915; highlighting a comparable mechanism of action for ERR activating autophagy.

As previously mentioned, the ERRs role in regulating cardiac autophagy has not been investigated. The few studies that link ERR to autophagy discuss ERR KO models resulting in decreased autophagy related gene expression but conclude that the link between ERR activation and autophagy is still unclear. To our knowledge, our data describe the first small molecule-mediated activation of ERR to increase expression of genes in the autophagy pathway. This adds to the field of ERR and autophagy by opening the door to pharmacologically targeting this receptor to enhance autophagy in diseases where this pathway is decreased.

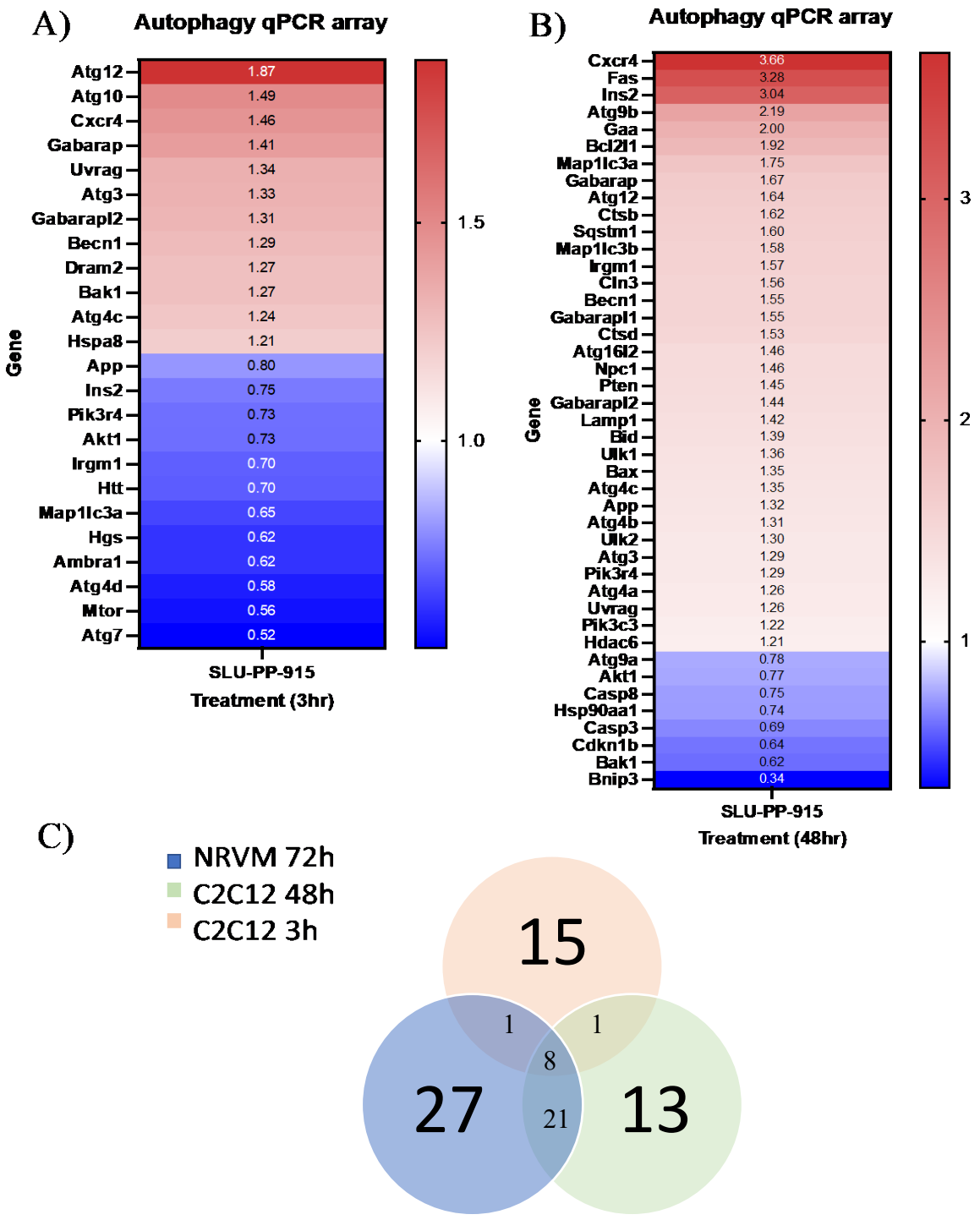

Figure 3.2: Genes involved with autophagy pathway are differentially expressed in C_2C_{12} cells treated with ERR agonist. C_2C_{12} cells were treated for A) 3 h or B) 48 h with SLU-PP-915 (5 μM) and gene expression measured by using an autophagy-focused qRTPCR array. C) A Venn diagram comparing NRVM (Fig. 11) and C_2C_{12} cells autophagy array gene expression shows overlap between the different cell types.

3.4.2 ERR Agonists Increase the Autophagy Flux in Cardiomyocytes

While expression of components of the autophagy pathway can be informative, functional measures of autophagy flux (autophagic degradation over time) is required to demonstrate the activity of this pathway. Several autophagic endpoints are typically monitored, such as autophagosome formation site, isolation membrane (or phagophore), and fusion with the lysosome.[7] In cultured cells one of the most common methods for studying autophagic flux is by using inhibitors of autophagy and then monitoring LC3 protein expression levels. LC3 is an autophagy marker that reflects the quantity of autophagosomes. LC3I is conjugated with phosphatidylethanolamine to form LC3II, that is localized to the autophagosome membrane. Bafilomycin A1 (BAF) is a V-ATPase inhibitor that blocks the fusion of the autophagosome to the lysosome to prevent degradation. This causes an accumulation of autophagy proteins like LC3II and p62, and if there is an increase in autophagy flux there will be a greater accumulation of these proteins with BAF treatment.

Since we observed robust increase in the expression of autophagy related genes after 72 h treatment, I performed an autophagy flux assay with both SLU-PP-915 and SLU-PP-332 at this time point. NRVMs were treated with SLU-PP-915 (5 μM) or SLU-PP-332 (10 μM) for 72 h, and then BAF (100 nM) was added at various time points (2-24 h). Both ERR agonists significantly increased autophagy flux over the BAF time course, measured by the abundance of p62 and LC3II protein and the 2hr BAF timepoint was shown (**Figure 3.3A-C**). This was also replicated at the 24 h treatment of SLU-PP-915 with BAF treatment at various time points (2-24 h) (data not shown). With an increase in autophagy flux at multiple timepoints and for two distinct compounds, we can

conclude that the ERR agonists are increasing autophagy both at the transcriptional level and in a functionally relevant manner.

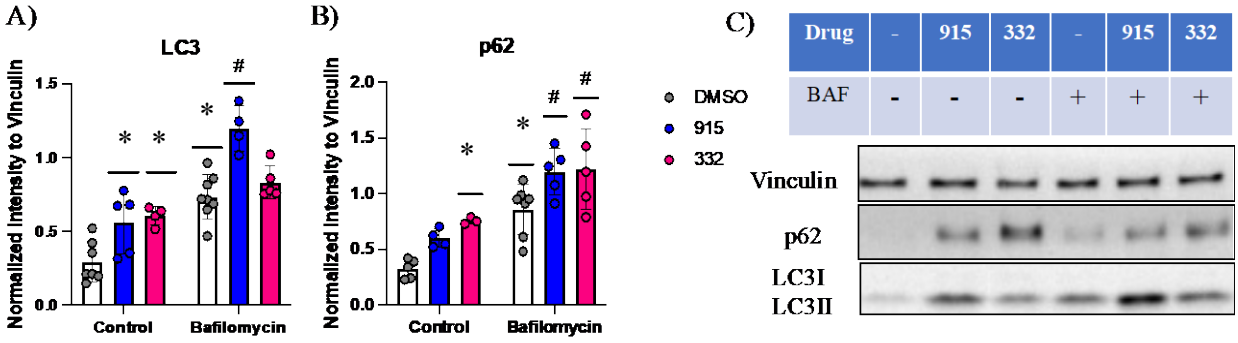

Figure 3.3: Autophagy flux increases with 72 h treatment of SLU-PP-915 or SLU-PP-332 at 2 h of BAF treatment. A) ERR agonists increase autophagy flux after 72 h treatment monitored by LC3 protein. B) ERR agonists increase autophagy flux after 72 h treatment monitored by p62 protein. C) Representative immunoblotting of data quantified in A-B).

3.4.3 ERR Agonists directly Regulate Expression of TFEB

Due to the important regulatory role TFEB plays in the autophagy-lysosome pathway, I sought to determine if an ERR agonist impacts the expression of this transcription factor. Since SLU-PP-915 induces autophagy more than SLU-PP-332 in all methods explored, SLU-PP-915 was used as the primary compound for the remainder of the experiments. SLU-PP-915 significantly increased TFEB mRNA and protein in NRVMs (**Figure 3.4A-C**). C_2C_{12} cells are immortalized murine myoblasts that are an excellent *in vitro* model to study the role of ERR in regulating TFEB because these cells overcome many pitfalls the NRVM model has. SLU-PP-915 significantly increased TFEB mRNA and protein in C_2C_{12} cells (**Figure 3.4D-F**).

Starvation is a known inducer of autophagy. Upon nutrient deprivation, ULK1 is dephosphorylated which allows separation from mTORC1 to initiate autophagy.[38] I compared the effect of SLU-PP-915 to starvation on TFEB activation. C_2C_{12} cells were treated for 72 h with SLU-PP-915 at 2.5 μM and acutely starved (24 h low glucose media). I observed similar levels of TFEB activation between the SLU-PP-915 and nutrient deprivation groups (**Figure 3.4G-H**). Several ERR targets were also assessed by qRTPCR in C_2C_{12} cells to validate this model (**Figure 3.4I-K**).

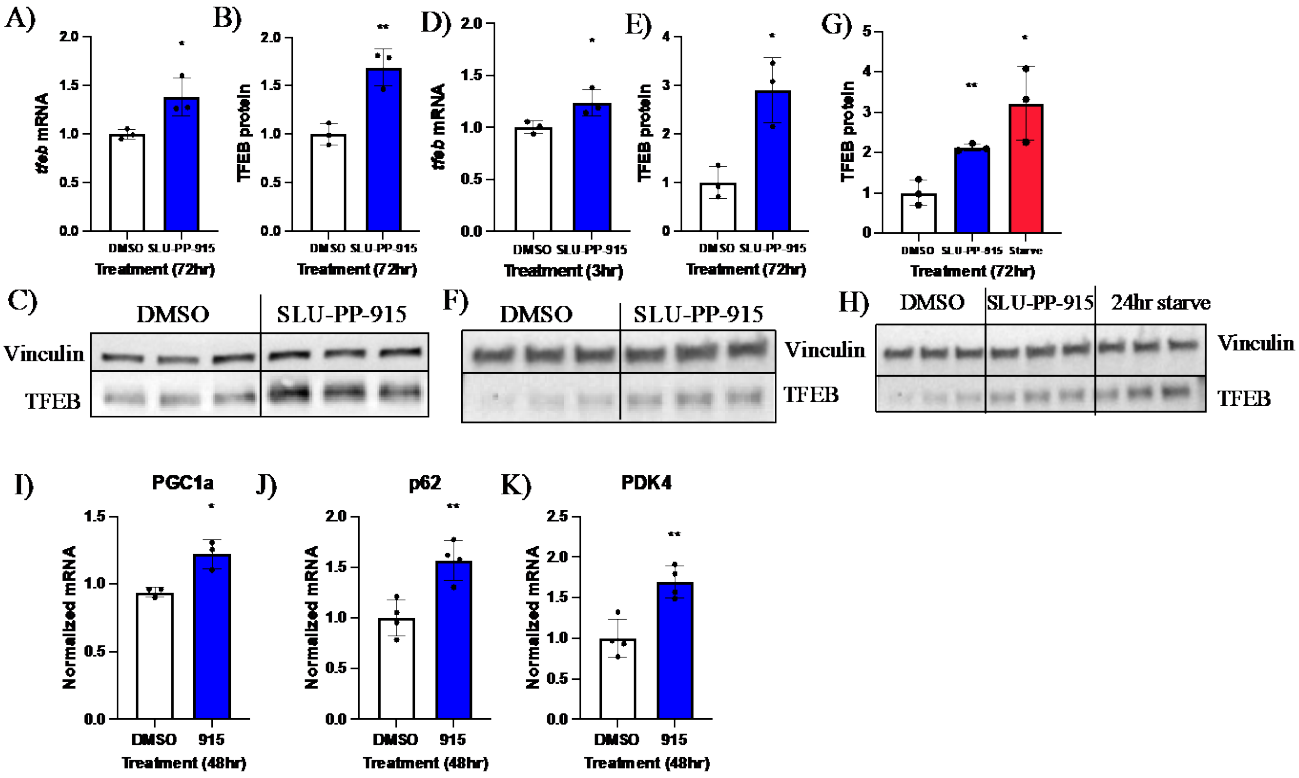

Figure 3.4: ERR agonists modulate autophagy genes and proteins. A) Gene expression of TFEB increases after 72 hr treatment with SLU-PP-915 (2.5 μM) in NRVMs. B) Protein expression of TFEB increases after 72 hr treatment with SLU-PP-915 (2.5 μM) in NRVMs. C) Representative immunoblotting of TFEB in NRVMs quantified in B). D) Gene expression of TFEB increases after 3 hr treatment with SLU-PP-915 (5 μM) in C_2C_{12} cells. E) Protein expression of TFEB increases after 72 hr treatment with SLU-PP-915 (5 μM) in C_2C_{12} cells. F) Representative immunoblotting of TFEB protein in C_2C_{12} cells quantified in E. G-H) TFEB protein expression was compared with SLU-PP-915 treatment (5 μM, 72 hr) and starvation (24 hr low glucose media). I-K) ERR target genes are significantly increased after 72 hr treatment with SLU-PP-915 (5 μM) in C_2C_{12} cells.

In a previous study, a ChIP-seq analysis for ERRγ was performed with ERRγ WT and KO human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) to identify direct ERR target genes.[39] We examined the TFEB promoter in this data set and found ERRγ occupancy in WT cells that was not present with the ERRγ KO samples (**Figure 3.5B**). This indicated ERRγ binding to the TFEB promoter but did not necessarily indicate transcriptional regulation. The sequence corresponding to the ChIP-seq peak was analyzed for ERRE sites using two transcription factor binding motif analysis tools: FIMO and MCAST.[40,41] We identified two potential ERRE sites in the TFEB promoter (**Figure 3.5A**). The TFEB promoter was cloned upstream of the luciferase gene resulting in a plasmid we named "TFEB-luc" to use in luciferase reporter assays. The TFEB-luc and expression vectors driving expression of full length ERRα, β, or γ were transfected into HEK293 cells, which were then administered increasing doses of SLU-PP-915. SLU-PP-915 treatment increased the luciferase signal in a dose response manner, demonstrating transcriptional activation (**Figure 3.5C-E**). Each isoform of ERR had a basal level of response when cotransfected with "TFEB-luc" that was enhanced with treatment of SLU-PP-915. The activity of TFEB-luc was analyzed as a function of nuclear receptor plasmid concentration (**Figure 3.5F-H).** As the concentration of nuclear receptor plasmid increased, the luciferase signals also increased. The activity of TFEB-luc was also assessed after treatment with several other ERR agonists (5 μM), which significantly increased the luciferase signal relative to DMSO treatment (data not shown).

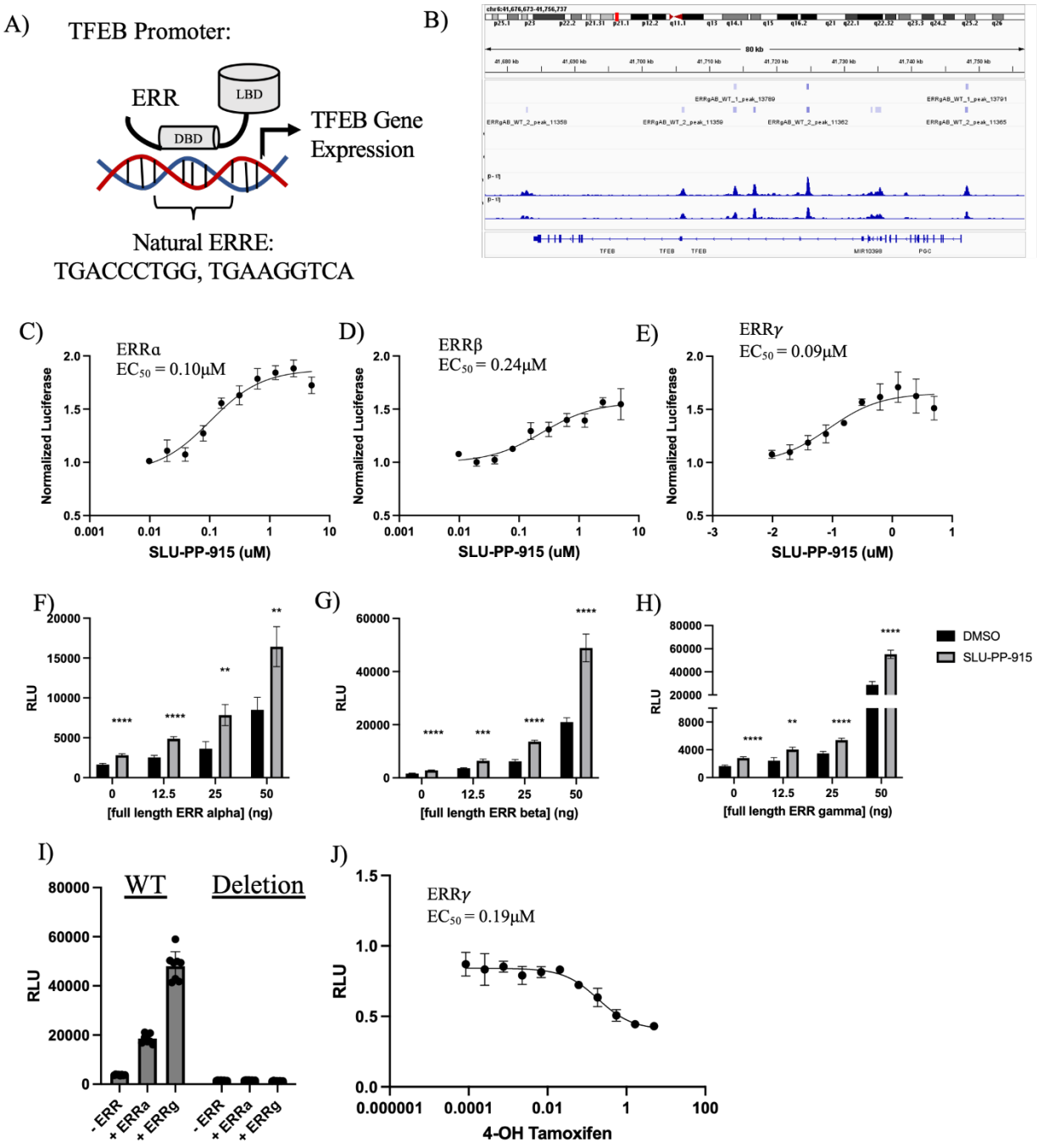

Figure 3.5: A) Graphic depiction of ERR binding to TFEB promoter. B) ChIP-seq of ERR γ from hiPSC-CMs reveal multiple peaks associated with the promoter of the TFEB gene. C-E) All three isoforms enhance TFEB promoter activity in a SLU-PP-915 dose-response manner. F-H) ERR expression vector dose response of luciferase signal with or without treatment of SLU-PP-915. I) Deletion construct with ERREs removed has significantly decreased luciferase activity compared with WT. J) ERRγ inverse agonist 4-hydroxy tamoxifen with TFEB-luc decreases luciferase signal in a dose response manner.

The next step was to delete the ERREs in the TFEB promoter to determine if those sites were required for ERR-mediated activation. The canonical ERRE is TCAAGGTCA and the found response elements in the promoter are TGACCCTGG and TGAAGGTCA. The region containing both response elements was deleted from the plasmid resulting in a new construct named "TFEB-deletion". TFEB-deletion exhibited significantly reduced the basal luciferase activity compared to TFEB-luc, consistent with ERR driving basal expression of the reporter (**Figure 3.5I**). Furthermore, the ability of ERR cotransfection to activate the TFEB-deletion reporter was completely abolished. These reductions in luciferase activity showed that the ERREs are required for transcriptional regulation of TFEB by ERRs.

An additional pharmacological approach was taken to confirm ERRs transcriptional regulation of TFEB by examining the effects of an inverse agonist on TFEB-luc activity. 4-hydroxy tamoxifen, an ERRγ inverse agonist, suppressed transcription driven by the TFEB reporter consistent with ERR mediated regulation of TFEB expression (**Figure 3.5J**).

TFEB must translocate to the nucleus to be in its transcriptionally active state. In the cytosol, TFEB is phosphorylated by mTORC1, AKT, GSK3β, ERK2, or MAPK4.[42] Upon activation by starvation or other stimuli, TFEB is dephosphorylated and shuttled to the nucleus. TFEB is actively exported from the nucleus by phosphorylation in response to stimuli such as refeeding (nutrient abundance).[43] To determine if SLU-PP-915 activates TFEB we performed a nuclear vs. cytoplasmic protein isolation and analyzed TFEB abundance by western blotting. After 72 h of treatment with SLU-PP-915 (5 μM) in C_2C_{12} cells, total and nuclear TFEB content

increased (**Figure 3.6A-B**). Torin was used as a positive control and significantly increased the nuclear TFEB levels (1 h treatment 250 nM). We also confirmed that SLU-PP-915 increased nuclear TFEB by using immunofluorescence microscopy. After 48 h treatment with SLU-PP-915, a significant increase in both total and nuclear TFEB staining was observed (**Figure 3.6C-H**).

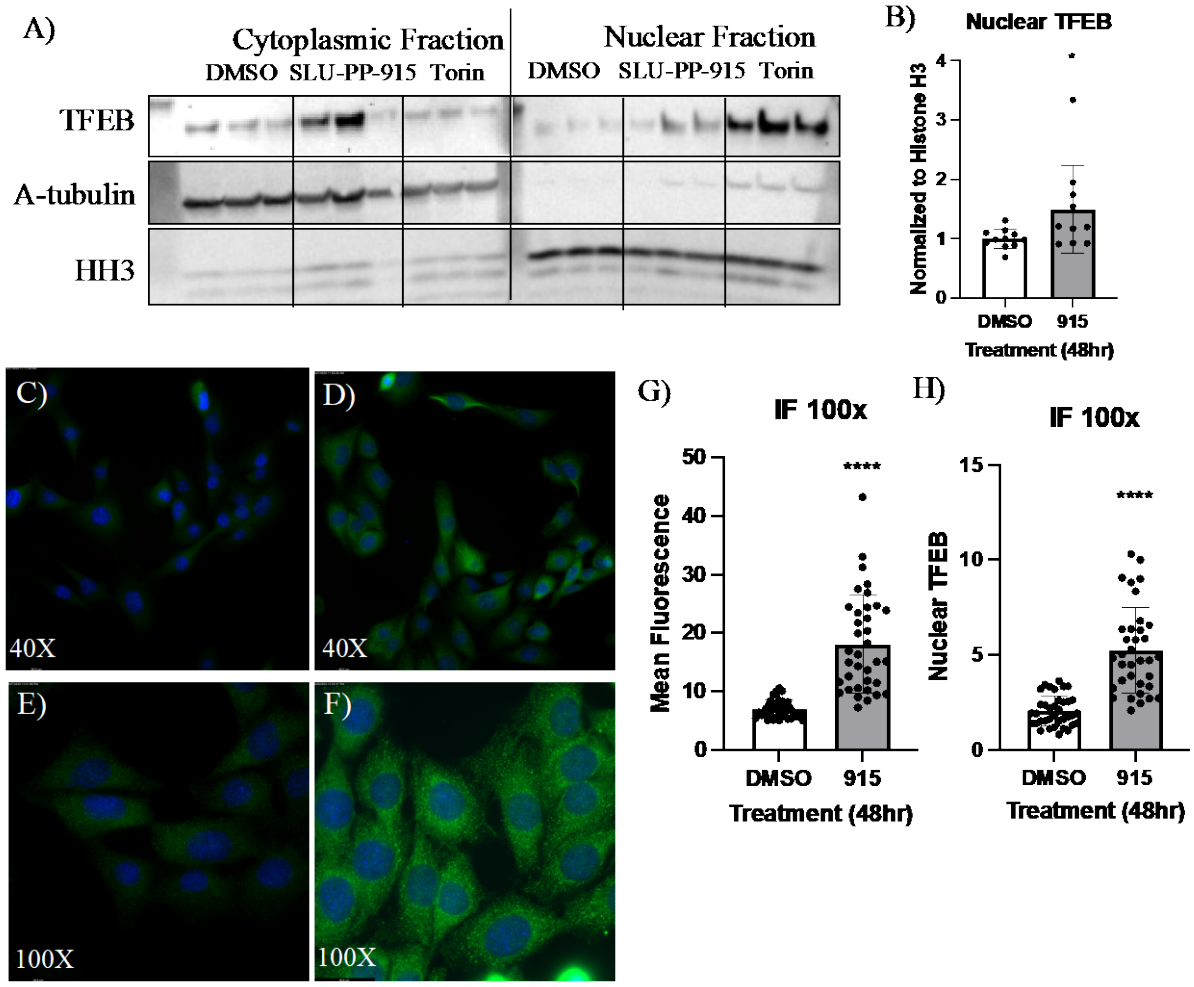

Figure 3.6: Total TFEB increases upon treatment with SLU-PP-915, but not the percent in the nuclear fraction. A) Immunoblotting of cellular fractionation of C_2C_{12} cells reveals 72 h treatment with SLU-PP-915 (5 μM) increases total TFEB protein, but not the percent of TFEB in the nuclear fraction. Torin (250 nM, 1 h) is used as a positive control. B) Protein abundances in A) quantified. C-D) Immunofluorescence staining of TFEB in C_2C_{12} cells treated for 48 hrs with DMSO or SLU-PP-915 (5 μM) at 40X. E-F) Immunofluorescence staining of TFEB in C_2C_{12} cells treated for 48 hrs with DMSO or SLU-PP-915 (5 μM) at 100X. G) Mean fluorescence of the whole cell is quantified utilizing imageJ software. H) Nuclear TFEB was quantified utilizing imageJ software.

Utilizing RNA-seq data discussed in Chapter 2, we compared the known target genes of TFEB to the genes modulated by SLU-PP-915 (**Figure 3.7A-C**). Importantly, NRVMs treated with SLU-PP-915 altered gene expression of several TFEB target genes in pathways such as autophagy/lysosome, transport, and metabolism. Several TFEB target genes that were identified as regulated in the RNA-seq data derived from treated NRVMs were validated in C_2C_{12} cells with qRTPCR (**Figure 3.7D-G**). Among these genes are *Ctsd, Mcoln1, Lamp1, and Lamp2. Ctsd* is a member of the cathepsin family that comprise a primary lysosomal protease.[44] *Mcoln1* is a Ca^{2+} channel located in the lysosomal membrane that assists in TFEB translocation by releasing calcium to activate the phosphatase calcineurin, which dephosphorylates TFEB.[45] *Lamp1* and *Lamp2* are membrane glycoproteins found in the lysosome that help maintain the acidic environment of the lysosome to facilitate degradation of cargo.[46] To further validate TFEB activation upon treatment of ERR agonist, I examined LAMP1 protein abundance and observed a significant increase after 72 h treatment of SLU-PP-915 (**Figure 3.7H-I**). Taken altogether, these data indicate that ERR activates the transcriptional activity of TFEB leading to increased autophagy.

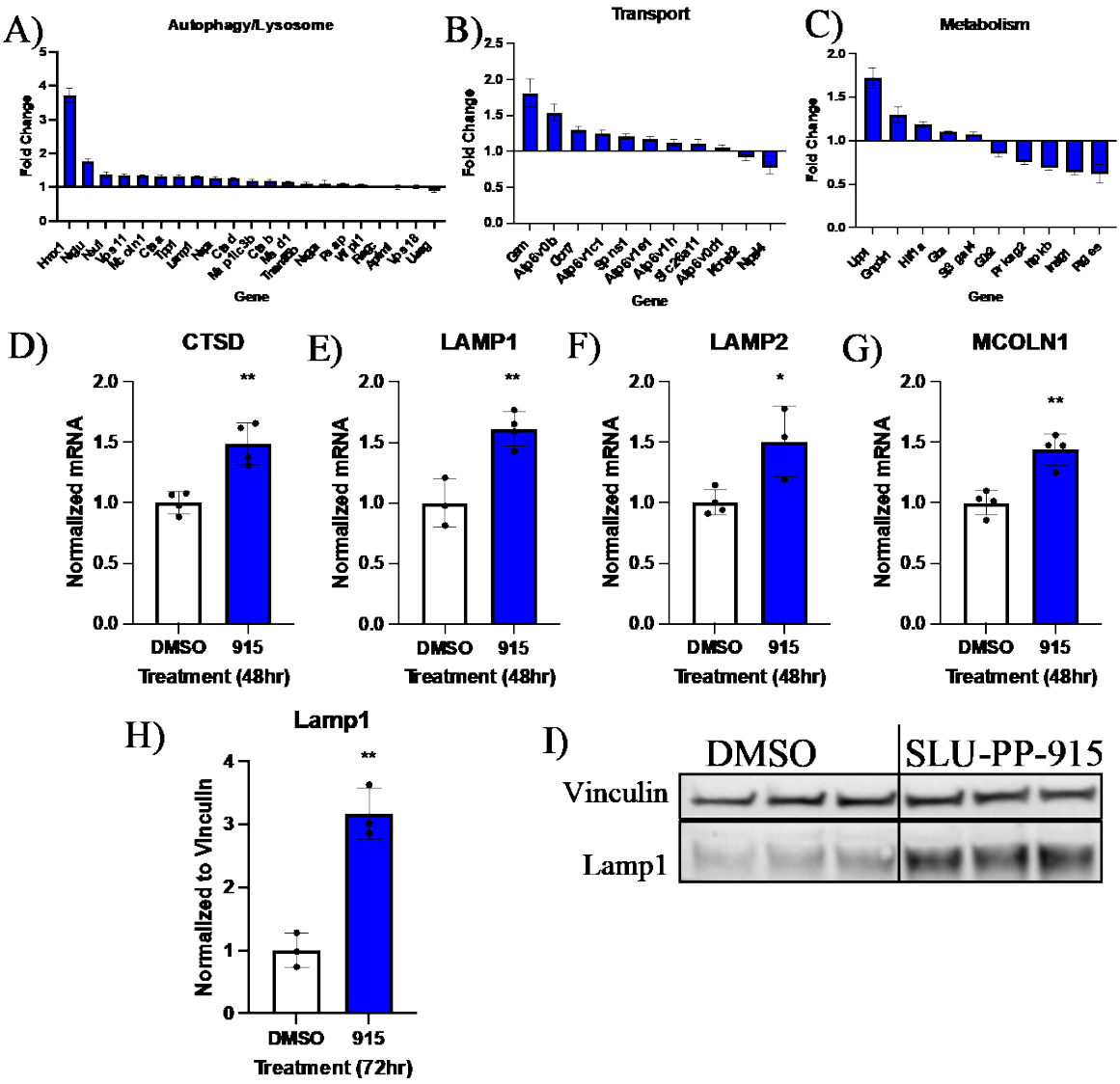

Figure 3.7: TFEB target genes are significantly increased upon treatment with ERR agonist SLU-PP-915. A-C) TFEB target genes are modulated in SLU-PP-915 treated NRVMs. The fold change is shown as SLU-PP-915 CPM/ DMSO CPM. D-G) SLU-PP-915 (5 μM) effects on TFEB target genes were validated with qRTPCR in C_2C_{12} cells treated for 48 h. H) Protein expression of Lamp1-TFEB target gene- increases after 72 h treatment with SLU-PP-915 (5 μM) in C_2C_{12} cells. I) Representative immunoblotting of Lamp1 in C_2C_{12} cells.

The observation that expression of TFEB target genes was modulated by SLU-PP-915 treatment suggests a signaling cascade whereby pharmacological activation of ERR increases transcription of the target gene, TFEB. Increased TFEB abundances facilitates increased TFEB target gene expression and enhanced autophagy. Our data suggest that ERR regulates transcription of TFEB. Interestingly, a recent study indicates that TFEB also directly regulates ERRα in the context of endometrial cancer.[47] This study reports TFEB increases transcription of ERRα in a luciferase reporter assay and confirmed TFEB directly binds to the promoter of ERRα with ChIP-qPCR. We propose a positive feedback mechanism that occurs between TFEB and ERR (**Figure 3.8**). Reciprocal regulation of ERR and TFEB may be important for maintaining cellular homeostasis.

These findings may have therapeutic utility for treating heart failure since we demonstrate that a pharmacologic agent that increases cardiac autophagy could potentially ameliorate the mitochondrial dysfunction, ROS, inflammation, and cardiomyocyte cell death. According to the CDC, 659,000 people die from heart disease each year in the United States, making it the leading cause of death.[48,49] In the TAC model of heart failure, autophagy was decreased relative to sham-mice, suggesting that an increase in autophagy may be cardioprotective.[23] There are no current treatments for heart failure that target cardiac autophagy and TFEB is a viable target for heart disease. Thus, there is interest in designing small molecule agonists for TFEB. Despite the plethora of positive results, there should be some caution in possible side effects resulting from increasing TFEB expression in excess. Cardiac specific overexpression studies of TFEB result in detrimental effects such as increased markers of pathological hypertrophy and premature death with evidence of heart failure due to decreased mitochondrial bioenergetic pathways, increased calcium

regulation and pro-fibrotic pathways.[50,51] Although overexpression studies have their advantages, their clinical relevance may not be realistic. In this case, increasing expression and transcriptional activity of TFEB would not be at the levels of overexpression, thus not cause the disadvantageous effects seen in that model.

ERR may be an ideal therapeutic target for treatment of heart failure since it not only targets autophagy-lysosome pathway as described in this Chapter, but also targets the metabolic dysfunction that occurs in the failing heart as described in Chapter 2. Genetic studies of cardiac specific ERRα/γ deletion in mice display heart failure phenotype with dysregulated metabolic, contractile, and conduction fucntion.[52] In heart failure, the shift in fuel utilization to glycolytic metabolism from oxidative metabolism contributes to disease progression. Thus, targeting ERR with a small molecule agonist will increase the oxidative metabolism machinery and reverse the metabolic pathways inhibited in the failing heart. By targeting both autophagic and metabolic deficits in heart disease, we can attack the disease progression from multiple angles.

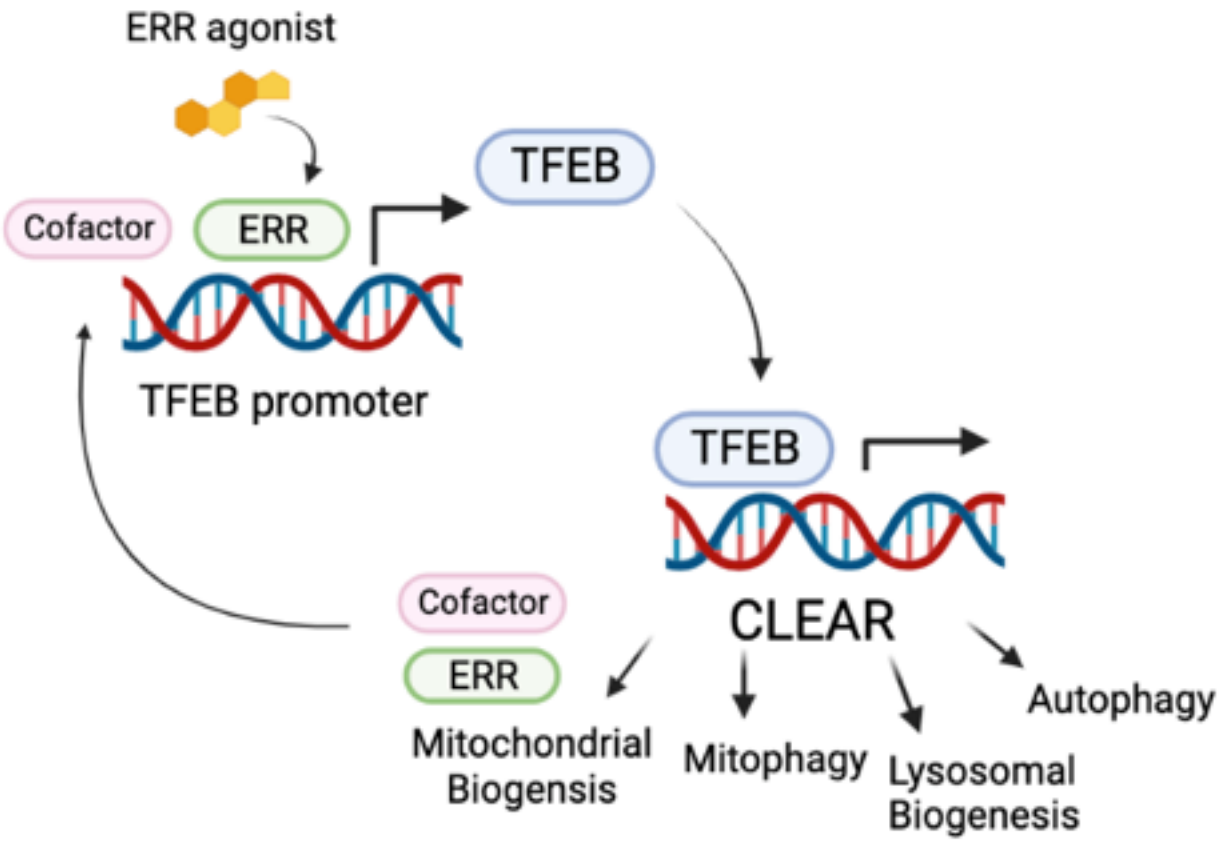

Figure 3.8: Proposed mechanism for ERR-TFEB regulation. Activating ERR with ERR agonist increases expression of TFEB through binding an ERRE in the TFEB promoter. TFEB regulates CLEAR gene network though binding its response elements. TFEB regulates gene pathways including autophagy, lysosomal biogenesis, mitophagy, and mitochondrial biogenesis. ERRs are transcribed by TFEB, thus completing the loop.

3.5 Conclusions

Based on our observation that ERR agonists increased expression of autophagy related genes, we hypothesized that ERR plays a critical role in transcriptional regulation of an array of genes that regulate this pathway. However, the exact mechanism (direct or indirect regulation) was not clear. We found that in NRVMs increased expression of autophagy genes upon ERR agonist

treatment and this was associated with increased autophagy flux. With treatment of SLU-PP-915, we observed significant increase in TFEB mRNA and protein in both NRVM and C_2C_{12} cells. This observation led us to hypothesize that TFEB may be a direct ERR target gene. We believe that increased TFEB, as a master regulator of autophagy gene expression, plays a role in the responsiveness to ERR agonists. We identified two putative ERREs in within the TFEB promoter using previously-published ChIP-seq where there was significant ERR occupancy. A TFEB promoter fragment containing these ERREs conferred ERR responsiveness to a luciferase reporter gene. Furthermore, when these ERREs were deleted, ERRs responsiveness was lost providing functional confirmation that ERR is regulating transcription of TFEB. Upon treatment with SLU-PP-915 the expression of TFEB increased, causing a greater concentration in the nucleus where it is in its active state. Since TFEB controls the transcription of many genes involved in the autophagy-lysosome pathway, this provides a novel mechanism for ERR control of autophagy. Future studies should determine whether inhibition of TFEB after ERR activation blocks the effects of SLU-PP-915 on autophagy. This knowledge opens the doors for studying ERR agonism for diseases with decreased autophagy.

3.6 Methods

3.6.1 Gene Expression Studies

For RT-PCR from NRVM or C_2C_{12} cell culture, RNA was extracted using RNeasy Mini Kit (Qiagen, 74106) per the manufacturers protocol. Reverse transcription was performed using iScript Reverse Transcription Supermix (BioRad, 1708891BUN). Sybr Green Supermix (Qiagen, 330523) was used for RT-PCR and results were analyzed in Quant Studio 5 Real-Time PCR System. Expression levels were normalized to PPIB (for rat) or 36B4 (for mouse) and the ΔΔCt-method.

3.6.2 Protein Expression Studies

Cells were lysed with RIPA buffer (VWR, 97063-270) with protease inhibitor cocktail (Sigma-Aldrich, 05056489001) and protein concentration determined with Pierce BCA assay kit (ThermoFisher Scientific, 23227). Samples were mixed with Tru-Page loading buffer (Sigma-Aldrich, PCG3009) and DTT before boiling for 5 min at 95ºC. Samples were loaded on 4-20% gels with TGS buffer (Fisher Scientific BP1341-1). Protein was transferred to PVDF membrane (Bio-Rad, 1704156EDU) and transferred with BioRad trans-blot turbo transfer system. The membrane was incubated in 5% nonfat milk in TBST for 1 h at room temperature. Primary antibody was diluted 1:1000 and incubations were overnight at 4ºC. The primary antibodies used were LC3 (Cell Signaling Technology, 12741S), p62 (Cell Signaling Technology, 5114S), TFEB (Cell Signaling Technology, 83010S), Lamp1 (Abcam, ab208943), alpha-tubulin (Abcam, ab15246), histone H3 (Cell Signaling Technology, D2B12), and Vinculin (Cell Signaling

Technology, 13901T). Secondary antibodies were diluted 1:3000 and incubated for 1 h at room temperature (Cell Signaling Technology, 7074S).

3.6.3 Autophagy Flux Analysis

NRVMs were seeded at 1.5 million cells per 6-well plate and treated for 72 h with ERR agonist. After 72 h treatment the cells were treated with bafilomycin A1 at 100 nM for 2, 4, 6, or 24 h. After the appropriate time, protein was extracted from the cells and western blot performed as described in 3.6.2. Protein markers LC3 and p62 were used to measure autophagy flux.

3.6.4 Reporter Assay

HEK293 cells were cultured in Gibco DMEM (ThermoFisher Scientific, 10566024) supplemented with 10% FBS at 37ºC with 5% CO_2. Cells were plated in 96-well plates at a density of $2x10^4$ in 50 μL media 24 h pre-transfection. The cells were transfected using Lipofectamine 2000 with 50 ng of reporter (TFEB-luc or TFEB-mutant-luc) and 50 ng of expression vector for ERR plasmids in 25 μL OptiMEM (Fisher Scientific, 31985088). 24 h post-transfection the cells were treated with 25 μL ERR agonist. On the final day of experimentation, 100 μL of Promega OneGlo Luciferase Reagent (Fisher scientific, PRE6130) was added to each well to lyse the cells. Luminescence values were normalized to DMSO control and data was analyzed using GraphPad Prism.

3.6.5 Nuclear Fractionation Assay

C_2C_{12} cells were plated at 200,000 cells/well in a 6-well plate and treated for 72 h with 5 μM SLU-PP-915. After 72 h, the cells for the positive control were treated with Torin for 1 h at 250 nM.

The nuclear fraction was separated from the cytosolic with Cellytic NuCLEAR Extraction Kit (Sigma-Aldrich, NXTRACT-1kt). Total protein in each fraction was quantified and protein abundance measured via western blotting methods as described in 3.6.2. Histone H3 was used as the loading control for the nuclear fraction and α−tubulin for the cytosolic fraction.

3.6.6 Immunofluorescence

C_2C_{12} cells were plated on coverslips in 12-well plates at 10,000 cells/well. After 24 h the cells were treated with SLU-PP-915 at 5 μM for 48 h. The cells were fixed with 4% paraformaldehyde (PFA) for 15 minutes then rinsed 3x with PBS. The cells were permeabilized with 0.1% Triton X-100 for 15 minutes, then rinsed 3x with PBS. The cells were blocked with 3% bovine serum albumin (BSA) in PBS for 1 h at room temperature. The cells were incubated overnight at 4ºC with TFEB antibody (1:200) (Fischer Scientific, 13372-1-AP). The following day the primary antibody was removed from the cells and rinsed with PBS 3x. Secondary antibody (1:1000) was incubated at room temperature for 1 h and the cells were rinsed 3x with PBS (Fisher Scientific, 111-545-144). The cells were incubated for 10 minutes with DAPI (1 μg/mL), then rinsed 3x with PBS. The coverslips were mounted with Prolong Gold Antifade (Invitrogen, P36934) overnight at room temperature. Slides were imaged with Leica Fluorescent Microscope and analyzed using imageJ software.

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Chapter 4: Conclusions and Future Directions

4.1 ERR agonists are a therapeutic candidate for targeting heart failure

Heart failure is a multifaceted disease that impacts millions of lives each year. There is an unmet need for novel treatments that target the disease from a different angle. Current treatments target symptom management, while activating ERR may target the source of disease progression. My thesis work looked at the beneficial impact of targeting ERR for the treatment of heart failure by two complimentary mechanisms: altering metabolic dynamics and autophagy.

Metabolic changes propagate heart failure by leading to cardiac remodeling. The change in fuel utilization from oxidative to glycolytic makes the myocardium less efficient, thus decreasing cardiac output, and increasing cardiac load. By restoring the oxidative capacity of cardiomyocytes, I hypothesized that we can halt disease progression, and potentially even improve disease state. In Chapter 2 of this work, I presented characterization of ERR agonists in both NRVM primary cardiomyocytes model and C_2C_{12} murine myoblast cell line. Through ERR activation, I observed increased gene expression of oxidative machinery, increased protein of ETC complex, increased fatty acid oxidation, increased mitochondrial content and activity, and increased respiratory capacity. These observations seen *in vitro* could be clinically beneficial at targeting the source cause of metabolic dysfunction in heart failure.

Due to changes that occur in cardiac remodeling, the cardiomyocytes have increased rates of cell death and decreased rates of autophagy. Without the autophagy pathway fully functional, cells accumulate proteins and cellular waste that becomes toxic and further propagate apoptosis. By increasing cardiac autophagy, I hypothesized that we can improve the health of cardiomyocytes to physiological state. In Chapter 3 of this work, I characterized the impact of ERR activation on autophagy in NRVMs and C_2C_{12} cells. I observed an increase of autophagy related gene expression, autophagy flux, and direct modulation of TFEB by ERR. Since ERR directly regulates the expression of TFEB, ERR agonists can cause a signal cascade of activating numerous genes in the autophagy-lysosome pathway. By targeting both the metabolic and autophagic dysfunction in heart failure, we aim to improve the disease in an additive manner.

4.2 Future Directions for determining efficacy of ERR agonists for heart failure

In vivo work has been done in parallel by our lab to study ERR agonists' impact on heart failure. A TAC model was used to mimic features of heart failure such as decreased left ventricular ejection fraction, hypertrophy, and fibrosis.[1] ERR agonists were found to reduce left ventricular systole volume, improve ejection fraction, and improve fractional shortening when compared to the TAC Vehicle and Sham treatments. ERR agonists significantly improved fibrosis and increased mitochondrial content. The morphological changes that occur after TAC surgery are profound: the cristae lose their distinct folds. After treatment with ERR agonists however, the morphology returns to normal and looks more like the Sham group. This work further emphasizes the role that ERR agonists play in mediating heart failure. A metabolomics study was performed to evaluate

alterations in metabolite composition from TAC and Sham models. It was determined that TAC treated with ERR agonist had a more similar metabolic profile to Sham group then to the TAC Vehicle group.

Since we observed such profound results in the TAC model of heart failure with our ERR agonists, a next step is to use this model to test the impact on autophagy markers in a diseased state. We can use the TAC model of heart failure and investigate key autophagy players gene expression, protein abundance, and flux. Several methods have been described to study autophagy flux *in vivo* such as injecting cloroquine before sacrificing the animals and monitoring LC3 and p62 proteins, and using RFP-GFP-LC3 reporter mice with immunofluorescence techniques to quantify autophagy flux.[2] A time course looking at autophagy after TAC will be important to understand where ERR agonists may be most beneficial since autophagy can increase in early phases of heart failure and decrease as the disease progresses.[3] Using this model would allow us to compare the results of our *in vitro* autophagy findings to the work previously done by the Burris lab *in vivo* on mitochondrial function and metabolism.

The TAC model mentioned previously looks at HFrEF, but an alternative next step would be to use a model that looks at the other form of heart failure- HFpEF. This form of heart failure is less studied and there are no good treatments for it due to the complexity and heterogeneity in nature.[4] There several models that could be used to study HFpEF *in vivo* such as leptin receptor deficient (db/db), leptin deficient model (ob/ob), high fat western diet, aging, and combination of models.[5] Since our ERR agonists target cardiometabolic function, the db/db or ob/ob model

combined with another risk factor element, such as aging, may be the first choice of models to best recapitulate the disease. Due to the rising prevalence and lack of therapeutics, it is important to study the potential of ERR agonists as a novel treatment for HFpEF.

Taken together with the results discussed in this thesis, there is strong supporting evidence for moving forward with these compounds preclinically. Preliminary pharmacokinetic/pharmacodynamic (PK/PD) studies have been performed by the Burris lab. SLU-PP-915 was dosed in mice at 5 mg/kg and has a half-life of approximately 16 minutes in the plasma and crosses the blood brain barrier. SLU-PP-332 was dosed in mice at 50 mg/kg b.i.d. i.p. and has a half-life of 1.8 h in the liver, 1.5 h in plasma, and 1 h in muscle. SLU-PP-332 does not cross the blood brain barrier. SLU-PP-915 seems to be the superior compound in both *in vitro* data and in PK/PD properties. Compounds are continuously being synthesized with SAR optimization and screened by the Burris lab to identify new hits that may surpass SLU-PP-915 performance.

Ultimately, the goal of any translational research is to identify novel therapeutics that end up being used in the clinic. Since both *in vitro and in vivo* studies have been completed, the next step for ERR agonists would be clinical trials. In phase 1 clinical trials, the drug candidate is tested in healthy volunteers to assess the safety of the compound. Phase 2 studies test a group of people with the disease being targeted and is evaluating proper dosing. Phase 3 clinical trials are the largest and often implemented in a double-blind study to assess the effectiveness at treating the disease target. The preclinical and clinical trial work is extremely rigorous to ensure the safest and most effective medicines are delivered to patients.

4.3 Future directions for determining ERRs mechanistic role in autophagy

There are several next steps to investigate ERRs role in inducing autophagy. Thus far, we know that ERR activates the autophagy pathway, but we do not know what stage of autophagy is activated. Analyzing the stage of autophagy- initiation, nucleation, autophagosome formation, autolysosome fusion, and lysosomal degradation- will provide a better mechanistic understanding of how ERR is activating autophagy flux. We have looked into acute starvation to activate autophagy, but additionally we can utilize mTOR inhibitors such as Rapamycin to induce autophagy in our system.[6] Conversely, autophagy can be inhibited by PI3K inhibitors.[7] AKT is a downstream protein of PI3K that I observed to be significantly decreased upon ERR agonism (Chapter 3), so studying ERRs role in the PI3K/AKT pathway of autophagic induction is of interest. To study phagophore formation, genetic knockout or knockdown of ATG genes can be used to elucidate ERRs role at this phase of autophagy.[8] Each of these experiments would allow us to determine whether ERR acts independently or dependent on the stage of the autophagic pathway that we are perturbing.

The studies thus far have focused on *in vitro* models using cardiac (NRVMs) and muscle cells (C_2C_{12}s), but understanding the impact ERR has on autophagy *in vivo* will give insight on the potential therapeutic impact that can be had. Beclin1 plays an important role in cardiac autophagy as seen by overexpression models increasing autophagy, mitophagy, and protecting the mitochondria while Beclin1 deficiency showed opposite characteristics.[9] Beclin1 was increased in each autophagy array dataset (Chapter 3), making it an interesting target to study further with

regard to ERR activation. Utilizing Beclin1 deficient mouse model with treatment of ERR agonists will allow investigation on if Beclin1 is crucial to ERRs activation of the autophagy pathway or if ERR is acting through an alternative pathway of activation.

Lastly, more studies can be done with regards to the ERR-TFEB axis. PGC1α is both a known coactivator of ERR and direct target of TFEB. Evaluating conditions with or without PGC1α and the impact on ERRs activation of TFEB will provide mechanistic insight and possibly a mode of feedback regulation. I hypothesize that PGC1α's abundance is increased in cellular conditions where autophagy is induced, leading to interaction with ERR and enhanced transcriptional control of TFEB. Additionally, TFEB knockdown or knockout models can be used to study ERR dependence on enhancing autophagy flux.

4.4 ERR agonists for other diseases

Looking away from heart failure, ERR agonists may have therapeutic utility for many other types of diseases. Firstly, ERR agonists may be useful in treating mitochondrial syndromes-inherited diseases that target the mitochondria.[10] Mitochondrial diseases do not have treatment that directly target the disorder, but rather help with symptom management.[11] There are many inherited mutations that can cause a mitochondrial disease, so the pathophysiology and presented symptoms will vary per person. Some mutations will cause issues with mitochondrial replication, respiratory chain assembly, transcription and translation of mitochondrial genes and proteins, protein import, and more.[12] A major goal of studying ERR agonists in this disease context will be to see what

desired therapeutic effects can we elicit and what portion of the effected population would benefit from this type of treatment. We have seen that ERR agonists have profound effects on mitochondrial health, so assessing their impact in this therapeutic context is of interest.

Neurodegenerative diseases are another target of interest that may have therapeutic utility from ERR agonists. I observed a significant decrease in SNCA, a gene heavily involved in diseases such as Parkinson's, Huntington's, and Alzheimer's. SNCA has been associated with Lewy bodies in the brains of patients with Parkinson's disease, so decreasing this protein may be a beneficial treatment.[13] Other members of the Burris lab have observed ERR agonism increasing neurogenesis in mice models with cognitive decline. Neurogenesis is the process of new formation of neurons that occurs in the hippocampus. During aging, neurons die and cannot be replaced, resulting in neurological impairment.[14] Increasing neurogenesis is thought to be a potential treatment for patients with cognitive decline. Taken together, studying ERR agonists in the context of neurodegenerative diseases may lead to fruitful findings and a therapeutic target not yet assessed.

4.4 Closing Remarks

Nuclear receptors account for a small portion of total human proteins yet are highly druggable and account for 16% of all approved small molecule drugs. The clinical success of this protein class makes them extremely exciting to continue studying for alternative therapeutic applications. The estrogen related receptor is not widely studied, yet from this thesis work shows great promise as a druggable target for both heart failure and beyond. This thesis expands the field

of nuclear receptors as pharmacological targets; specifically, on ERR as a viable target for heart failure.

4.4 References

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