ERRβ SPLICING VARIANTS DIFFERENTIALLY REGULATE CELL CYCLE PROGRESSION

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ABSTRACT

Orphan receptors comprise nearly half of all members of the nuclear receptor superfamily. Despite having broad structural similarities to the classical estrogen receptors, estrogen-related receptors (ERRs) have their own unique DNA response elements and functions. In this study, we focus on two ERRβ splice variants, short form ERRβ (ERRβsf) and ERRβ2, and identify their differing roles in cell cycle regulation. Using the acyl hydrazone DY131 (a synthetic agonist of ERRβ) as a tool to modulate endogenous ERRβ function, splice-variant selective shRNA, and exogenous ERRβsf and ERRβ2 cDNAs, we differentiate between splice variant function in cell cycle regulation. We demonstrate the role of ERRβsf in mediating the G1/S checkpoint through p21. We also show ERRβsf is required for DY131-induced cellular senescence. A key novel finding of this study is that ERRβ2 can mediate a G2/M arrest. Furthermore, in the absence of ERRβ2, the G2/M arrest is reversed and ERRβsf now induces p21 and initiates a G1/S arrest. These data imply a potential dominant inhibitory role for ERRβ2 on ERRβsf. Furthermore, we demonstrate DY131 cytotoxic sensitivity is dependent on p53 status. In the absence of wild-type p53 (null or mutated), activated ERRβ2 initiates an apoptotic response. In wild-type p53, ERRβsf induces p21 in a cytoprotective response and the magnitude of cell death is lessened. This study illustrates both novel functions for ERRβ splice variants as well as evidence for splice variant interaction.
DEDICATION

I dedicate this thesis to my husband, Mike, who has feigned more interest in this project than anyone I know. If it weren’t for his love, support, humor and encouragement, this process would have been much, much harder.

I would also like to dedicate this work to my mom, Maureen, and in loving memory of my dad, Carmen, without whom I would not be the person I am today.
Now all glory to God, who is able, through his mighty power at work within us, to accomplish infinitely more than we might ask or think. Glory to him in the church and in Christ Jesus through all generations forever and ever. Amen!

Ephesians 3:20-21

Above all, I would like to express my deepest gratitude to the Lord who created all things far superior and far more complex than any hypothesis I could postulate. Without His generosity, grace, and the gifts He has blessed me with, I would not have been able to uncover this small piece of His greater work.

I sincerely thank my mentor, Dr. Rebecca Riggins, for her patience throughout my training and for generating a phenomenal lab environment that allowed me to grow into an independent scientist. I appreciate her dedication to my training and investment in my project.

I thank all of my committee members, Drs. Christopher Albanese, Maria Laura Avantaggiati, Ian Gallicano and Anton Wellstein, who sacrificially invested in my project. Without their insights, the project would not have become the body of work it is today. I greatly appreciate their time, energy, and willingness to help.
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>5-FU</td>
<td>Fluorouracil</td>
</tr>
<tr>
<td>AIP1</td>
<td>Apoptosis-Inducing Protein 1</td>
</tr>
<tr>
<td>BAD</td>
<td>Bcl-2-associated death promoter</td>
</tr>
<tr>
<td>BAX</td>
<td>Bcl-2-associated X protein</td>
</tr>
<tr>
<td>BBC3</td>
<td>BCL-2 binding component 3</td>
</tr>
<tr>
<td>Bcl-xl</td>
<td>B-cell lymphoma extra large</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>BID</td>
<td>BH3 interacting domain</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast cancer 1</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>Cip/Kip</td>
<td>CDK interacting protein/Kinase inhibitory protein</td>
</tr>
<tr>
<td>CKI</td>
<td>cyclin-dependent kinase 2</td>
</tr>
<tr>
<td>c-Fos</td>
<td>FBJ murine osteosarcoma</td>
</tr>
<tr>
<td>CAR</td>
<td>constitutive androstane receptor</td>
</tr>
<tr>
<td>CTE</td>
<td>Carboxy-terminal extension</td>
</tr>
<tr>
<td>Cyt C</td>
<td>Cytochrome C</td>
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<tr>
<td>DDB2</td>
<td>DNA damage-binding protein 2</td>
</tr>
<tr>
<td>DR</td>
<td>Death receptor</td>
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<tr>
<td>DRAM1</td>
<td>DNA-damage regulated autophagy modulator 1</td>
</tr>
<tr>
<td>DY</td>
<td>DY131</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
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Erk Extracellular signal-regulated kinase
ERE Estrogen response element
ERR Estrogen-related receptor
ERRE Estrogen-related response element
ERRβsf short form ERRβ
ESC Embryonic stem cell
ESE Exonic splicing enhancer
ESS Exonic splicing silencer
FADD Fas associated death domain
Fas First apoptosis signal
FasL Fas ligand
FITC Fluorescein isothiocyanate
FXR Farnesoid X receptor
GADD45A Growth arrest and DNA-damage-inducible protein
GBM Glioblastoma multiforme
GFP Green fluorescent protein
GOF Gain-of-function
G1 Gap 1
G2 Gap2
hnRNP heterogenous nuclear ribonucleoprotein
iPSC induced pluripotent stem cell
ISE Intronic splicing enhancer
ISS Intronic splicing silencer
c-jun Jun proto-oncogene
LOF Loss-of-function
LXR Liver X receptor
MAPK Mitogen activated protein kinase
MDM2 Murine double minute 2
MEF Mouse embryonic fibroblasts
MEK Mitogen activated protein kinase kinase
NLS Nuclear localization sequence
NOXA Phorbol-12-myristate-13-acetate-induced protein 1
NR Nuclear receptor
ORF Open reading frame
PAI-1 Plasminogen activator inhibitor-1
PALA N-(phosphoacetyl)-L-aspartate
PARP Poly ADP ribose polymerase
PCD Programmed cell death
PCNA Proliferating cell nuclear antigen
PMAIP1 Phorbol-12-myristate-13-acetate-induced protein 1
Pol II Polymerase II
PPAR Peroxisome proliferator-activated receptor
pRb Retinoblastoma protein
PXR/SXR Pregnane X receptor/sensing nuclear receptor
RXR Retinoid X receptor
SR Ser/Arg-rich proteins
SRp Splicing regulatory protein

TAM Tamoxifen

TNFR1 Tumor necrosis factor receptor 1

TRAIL Tumor necrosis factor-related apoptosis-inducing ligand

UV Ultra violet

WAF1 Wild-type p53 activating fragment 1
CHAPTER 1

INTRODUCTION
INTRODUCTION

A largely growing field of research is focusing on the emerging role of alternative splice variants in disease progression. Alternative splicing occurs in almost 95% of all mammalian genes (1) and is a key determinant in the diversity of the proteome. Defects in alternative splicing can dramatically alter the protein profile of a cell and have been directly linked to a variety of human diseases: β+-thalassemia (2,3), spinal muscular atrophy (4), acute myeloid leukemia (5), hepatocellular carcinoma (6), and others.

The Riggins’ lab studies the role of orphan nuclear receptors, specifically the estrogen-related orphan nuclear (ERR) family, in cancer. The ERR family has three isoforms: α, β, and γ. Previously, we have investigated the role of ERRγ in mediating drug resistance in breast cancer. Of the three ERRs, the least is known about ERRβ. ERRβ has three splice variants. Given the impact of alternative splicing on the proteome and disease progression, this project focused on understanding the endogenous function of ERRβ splice variants in cancer globally where little research has been conducted.

1.1 PROGRAMMED CELL DEATH: APOPTOSIS

When a cell determines it should die rather than continue to replicate, it undergoes some form of programmed cell death (PCD). The type of PCD depends on the stimuli, cell type, functional signaling pathways available and other factors. Even with those determinants, crosstalk still exists between different modes of death. One form of PCD, apoptosis, can be initiated by either external (extrinsic pathway) or internal (intrinsic pathway) cues. In the extrinsic pathway, extracellular ligands (TRAIL, FasL) bind death receptors (Fas, TNFR1, DRs, etc.) on the cell surface. This binding recruits adaptor molecules (FADD) to the intracellular
death domain of the receptor, where the adaptor molecule activates initiator caspases, caspase -8 or -10. Initiator caspases then activate effector caspases, caspases -3, -6, or -7 (7) (Fig 1). The effector caspases are highly specific proteases that activate nucleases and destroy cellular structure, ultimately killing the cell (8).

The mitochondria play a critical role in the intrinsic pathway. The balance between Bcl-2 family members controls the permeability of the outer mitochondrial membrane. Anti-apoptotic members, Bcl-2 and Bcl-XL, are located at the mitochondrial membrane while pro-apoptotic members, BAD, BAX, BID, are mainly in the cytosol (7). In response to an apoptotic stimulus, the pro-apoptotic members translocate to the mitochondrial membrane where they form pores on the mitochondria. These pores allow for the release of cytochrome C (Cyt C). Once in the cytoplasm, Cyt C complexes with Apaf-1 and caspase 9 to form the apoptosome which can activate the same effector caspases as the extrinsic pathway (Fig 2). The extrinsic and intrinsic pathways can converge at different stages of apoptosis. For example, activation of caspase 8 can lead to Bid translocation to the mitochondrial membrane and release of Cyt C (9).

p53 is a major regulator of both the extrinsic (Fas, DR4, DR5, caspase-8 induction) and intrinsic (Bid, Bax, Noxa and BBC3 induction) apoptotic pathways (10). The role of p53 in apoptosis is to act as a sensor to DNA damage and prevent genetically unstable cells from continuing to proliferate. This is one reason p53-null mice have a higher incidence of tumors (11).

1.2 CELLULAR SENESCENCE

1.2.1 Permanent cell cycle arrest: senescence
Figure 1. Extrinsic apoptosis signal transduction pathway (adapted from (12)).
Figure 2. Intrinsic apoptosis signal transduction pathway (adapted from (12)).
Senescence is a permanent cell cycle arrest that prevents a damaged cell from continuing to proliferate. It therefore is an important part of tumor suppression and plays a fundamental role in aging. A damaged cell could mean a variety of conditions: an older cell that has reached its Hayflick limit, a cell that has experienced genotoxic stress, extensive DNA damage, or irreparable mutations (13,14). Most frequently, a senescent cell has DNA content of G1 phase, although in certain genetic backgrounds, other DNA content arrests occur (15). Despite the irreversible growth arrest, the cell remains metabolically active. Morphologically, the cell looks flattened and hypertrophic (enlarged) (16).

Commonly, but not always, a transformed cell line has developed mechanisms to bypass senescence and is able to replicate indefinitely in culture. At the same time, certain stressors, such as anti-cancer therapies, are able to induce senescence in a transformed cell line (17).

Although cells are mechanistically competent to undergo apoptosis and senescence, the two events are typically mutually exclusive. Once a cell senesces, it often becomes resistant to apoptosis, even when chemically manipulated with agents designed to induce an apoptotic response (15). Therefore, it is likely crosstalk exists between the two cellular responses. It has been demonstrated that p53, a common mediator of both responses, can be selectively recruited to one pathway and therefore unavailable to activate the other response (18).

1.2.2 Pathways involved in senescence

The two major, but not exclusive, pathways mediating senescence are the p53-p21 and p16-pRb pathways (15). The p53-p21 signaling pathway also initiates a more transient cell cycle arrest (G1/S), however the molecular switch dictating senescence versus G1/S arrest is currently
unknown. One potential determinant could be the rate of DNA repair. For example, prolonged or delayed repair could signal the permanent arrest phenotype whereas faster machinery is able to achieve successful repair while replication is only temporarily stalled and senescence is avoided (15). Although DNA damaging events often trigger the p53-p21 pathway first, they are also able to activate the p16-pRb pathway (19). pRb can inactivate E2F, a transcription factor critical for cell cycle progression, leading to the repression of proliferative genes such as c-FOS, cyclin A, cyclin B, and proliferating cell nuclear antigen (PCNA) (20-22).

1.3 TUMOR SUPPRESSOR: p53

1.3.1 p53 function

The TP53 gene is the most commonly mutated gene in all cancers, with over 50% of human cancers harboring an inactivating mutation (23). The stability and overall levels of the p53 protein in a cell dictate its function. Under normal cellular conditions, the p53 protein has a relatively short half-life (5-10 min) (24), is negatively regulated by E3 ubiquitin ligases, namely MDM2, (25,26) and maintained at low levels in the cell. In response to cellular stress, p53 can activate different target genes leading to various cell fate outcomes: cell cycle arrest (CDKN1A), apoptosis (BAX, BBC3, PMAIP1), autophagy (DRAM1) DNA repair (BRCA1, GADD45A, DDB2), senescence (PAI-1), and others (27-31).

1.3.2 p53 regulation by posttranslational modifications

Posttranslational modifications play a major role in p53 function. For example, phosphorylation at S46 is critical for initiating an apoptotic response (specifically through
activation of p53-regulated AIP1), but not cell cycle arrest (32,33). Phosphorylation can also affect the stability of the p53 tetramer. After exposure to ultra violet (UV) light, S392 is phosphorylated and this increases the stability of the p53 tetramer. Other posttranslational modifications such as acetylation also impact p53 function and stability. Acetylation of K320 promotes cell cycle arrest through p53-mediated induction of p21 (34). In a mouse model, mutation of the equivalent lysine (K317R) causes an increase in pro-apoptotic gene expression after UV exposure (35).

1.3.3 Wild-type versus mutant p53 functions in cancer

Most frequently when p53 is mutated in cancer, the mutation is a single nucleotide substitution within the DNA-binding domain (26). The selective advantage for retaining mutant p53 varies between cancer cells. Mutant p53 can have either gain-of-function (GOF) or loss-of-function (LOF) properties. In a GOF scenario, the mutant protein can acquire oncogenic properties such as activating novel target genes involved in proliferation, angiogenesis and survival (36). At the same time, mutations in p53 can impair the protein’s ability to bind the promoters of its normal tumor suppressive target genes or act as a dominant negative in the tetramer complex with wild-type p53 (37).

Given the pervasiveness of p53 mutations in cancer, much research has been dedicated to understanding the therapeutic consequences of p53 inactivation. Conflicting data exits regarding whether p53 inactivation leads to heightened drug sensitivity or drug resistance. At least part of the confusion could be explained by mutant p53 having both GOF and LOF capabilities as discussed above. Therefore, evidence supports both possibilities.
To avoid confounding variables and more directly elucidate the specific role of p53 in drug responsiveness, isogenic variants and mouse embryonic fibroblasts (MEFs) are frequently used. In a colon cancer model, using isogenic p53 variants of HCT116 (p53<sup>+/−</sup>) cells, Bunz et al. demonstrate sensitivity to different DNA-damaging agents based on p53 status depends on the drug. For example, Adriamycin treatment induces apoptosis in p53<sup>−/−</sup> cells, but not p53<sup>+/−</sup>. In contrast, 5-FU treatment leads to apoptosis in p53<sup>+/+</sup> cells while p53<sup>−/−</sup> cells are resistant to apoptosis (38).

This study also tested whether drug sensitivity patterns were preserved in a xenograft model. After treating animals with γ-irradiation, they observed no difference in tumor growth when comparing p53<sup>+/+</sup> and p53<sup>−/−</sup> mice. Despite this negative result with γ-irradiation treatment in vivo, they found treatment with 5-FU was consistent with their in vitro experiments. They observed significant tumor regression in the p53<sup>+/+</sup> mice, but not in animals where p53 was absent.

1.3.4 p53-independent apoptosis

As mentioned above, p53 is a major sensor in the cell and is able to convert various stress signals into appropriate responses for the cell: cell cycle arrest or cell death. Tissue type can dramatically impact the cellular response to the same damaging event. For example, one study investigated the responsiveness of prostate stromal cells (fibroblasts) and the adjacent epithelial cells to γ-irradiation (39). Interestingly, the stromal cells activate p53 and arrest whereas the epithelial cells do not induce p53 or undergo cell cycle arrest.

Although less common than the canonical apoptotic response, mechanisms of p53-independent apoptosis do exist. Aladjem et al. show that undifferentiated murine embryonic stem
(ES) cells exposed to DNA damaging agents undergo apoptosis, regardless of p53 status (40). Using homologous recombination, they treated p53<sup>+/+</sup> and p53<sup>-/-</sup> ES cells with increasing concentrations of Adriamycin and measured cell survival and TUNEL staining. They found both cell populations had similar levels of apoptotic cells, despite differences in p53 expression. In the p53<sup>+/+</sup> cells, they found that p53 is primarily cytoplasmic and bound to MDM2. Overexpression of p53 could not rescue an apoptotic response therefore other factors downstream of p53 likely contribute to the inability to mount an apoptotic response in ES cells. These data illustrate a role for apoptosis, independent of p53 status.

1.4 CYCLIN-DEPENDENT KINASE INHIBITOR: p21

1.4.1 p21 and cell cycle regulation

p21 was initially discovered as a transcriptional target of the tumor suppressor, p53 (28). Concurrently, p21 was also shown to bind cyclin-dependent kinase 2 (CDK2) using a yeast-two hybrid screen (41). p21 belongs to the Cip/Kip cyclin-dependent kinase 2 (CKI) family which include p27 and p57 (42). While p21 shares significant sequence homology with CKI family members in its N-terminus, it has a unique carboxyl-terminal that allows it to also interact with the proliferating nuclear antigen (PCNA) subunit of DNA polymerase δ (43).

Maintaining genomic integrity is a fundamental goal of the cell. Therefore, precise checkpoints exist throughout the cell cycle to ensure DNA is replicated without mutations and abnormal cells cannot proliferate. p21 is a major regulator of the G1/S checkpoint, preventing inappropriate DNA replication in S phase. In response to various stimuli (oxidative stress, DNA damaging agents), p21 can become activated. Once activated, p21 binds both the cyclin and
CDK subunits of the cyclin/CDK complexes, primarily through its N-terminus (44). p21 can recognize, bind and inhibit many of the cyclin/CDK complexes: cyclin E/CDK2, and cyclin A/CDK2,1. This inhibition prevents the phosphorylation of the retinoblastoma tumor suppressor, pRb. Without hyperphosphorylation, Rb remains bound to the transcription factor, E2F. E2F is no longer able to bind target genes required for DNA synthesis and the cell undergoes a G1/S arrest (Fig 3).

Using a p21 isogenic mutant colon cancer model, Waldmann et al. have shown that p21 is necessary for p53 mediated G1 arrest after DNA damage (45). Using HCT116 cells, which are p53 and p21 wild-type and undergo a G1/S arrest after exposure to DNA-damaging agents, the Vogelstein lab used homologous recombination to generate a HCT116 variant with p21 deleted, HCT116p21−/−. When they treated the HCT116p21+/+ parental cells with either ionizing radiation or Adriamycin, they observed a p53 and p21 induction and G1/S arrest, consistent with other reports in the literature (46,47). In contrast, in the HCT116p21−/− cells, they observed only a p53 induction and a G2/M arrest. These data demonstrate p21 is downstream of p53 and necessary for a G1/S arrest after p53 activation with Adriamycin. These findings are consistent with Deng et al. who demonstrated that p21 is required for G1 arrest after exposure to N-(phosphoacetyl)-L-aspartate (PALA) using a p21−/− MEF model (48).

1.4.2 p21 protein expression levels and function

p21 is a complex protein with a variety of biological roles, sometimes in opposition of one another. As discussed above, p21 is commonly recognized for its antiproliferative role through inhibition of cyclin-CDK complexes. Interestingly, p21 has also been shown to activate cyclin/CDK complexes dependent on p21 expression levels (49). Using p21−/− and p27−/− MEFs,
Figure 3. The role of p21 in G1/S cell cycle arrest (adapted from (50)).
Cheng et al. demonstrate that upon stimulation with mitogen factors, low levels of p21 and p27 are required for proper assembly of cyclin D/CDK complexes. They conclude p21 expression levels dictate whether it is an inhibitor or activator of the cyclin/CDKs.

1.4.3 Posttranslational modifications and p21 localization

In addition to p21 protein levels, posttranslational modifications and cellular localization have been shown to regulate p21 function. Phosphorylated p21 at Thr145 interferes with the protein’s nuclear localization sequence (NLS) and consequently redirects p21 to the cytoplasm. Cytoplasmic localization prevents p21 inhibition of cell cycle regulatory proteins and has been shown to lead to oncogenic properties (16).

1.4.4 p21 and apoptosis

p21 has also been shown to play a role in suppressing apoptosis. N-butyrate, a naturally occurring cancer preventative fatty acid in the colon, induces apoptosis in a p21-independent manner (51). HCT116 p53 and p21 isogenic variants were treated with physiological concentrations of n-butyrate, and the fraction of apoptotic cells were measured. Although there was no difference in apoptosis between p53+/+ and p53−/− cells, p21−/− cells showed a significant increase in apoptosis compared to p53 variants (51). The cytoprotective effect of p21 was confirmed through exogenous re-expression of p21 into p21−/− cells leading to a significant decrease in apoptotic cells. Likely, without stimulation of 21 and a subsequent G1/S arrest, the cell progressed through S phase with damaged DNA and ultimately succumbed to PCD after failing later checkpoints. Interestingly, this study also showed an increase in mitochondrial
proliferation (through electron micrographs and mitochondrial number) closely correlated with the magnitude of the apoptotic response.

Mechanistically, Shaulian et al. discovered c-jun is able to modulate the p53-p21 promoter interaction. After UV irradiation, p53 binds the p21 promoter transiently, but dissociates by 6h post exposure in c-jun\(^{+/+}\) MEFs, followed by apoptosis (52). In contrast, p53 remained bound to the p21 promoter for longer than 24h post UV exposure in c-jun\(^{-/-}\) MEFs. Furthermore, the prolonged p53-p21 interaction in the absence of c-jun led to increased p21 expression and cell cycle arrest, but not apoptosis. Therefore, p21 expression and the subsequent cell cycle arrest led to cytoprotection and inhibition of apoptosis.

**1.5 ALTERNATIVE SPLICING**

**1.5.1 Alternative splicing regulation**

A single gene can give rise to hundreds, or even thousands, of different gene products. This occurs through the intricate processing of an mRNA transcript known as alternative splicing. Alternative splicing allows for different exons to be excluded or introns included, generating vastly diverse transcripts that ultimately are translated into different proteins. Alternative splicing occurs in almost 95% of all mammalian genes (1,53) and is orchestrated by a complex group of ribonucleoproteins known collectively as the spliceosome (54). [Figure 4](#) details the intricate complexity of splicing. The spliceosome recognizes specific splice site consensus sequences within introns and these recognition sites are considered either strong or weak depending on their sequence homology to the consensus sequence (54). Alternative splicing robustness is also governed by other sequences within both introns and exons that recruit...
Figure 4. Complexity of RNA splicing (adapted from (55)).
splicing regulatory proteins that act on the spliceosome as enhancers (exonic/intronic splicing enhancers, ESE/ISE) or silencers (exonic/intronic splicing silencers, ESS/ISS) (56). There are two main splicing regulatory protein families: Ser/Arg-rich proteins (SRs) and heterogenous nuclear ribonucleoproteins (hnRNPs) (57,58) and they have been shown to bind both exons and introns as silencers and enhancers (59).

RNA structure also impacts splicing by affecting the accessibility of consensus sequences by the spliceosome and/or regulatory proteins (60). Current research illustrates splicing can occur simultaneously with transcription, co-transcriptional splicing, or after transcription is complete, post-transcriptional splicing (61). Therefore, whether the secondary and tertiary structure of the transcript has formed yet is highly relevant to splicing. Additionally, it has been shown that the transcriptional machinery also impacts recruitment of splicing factors and affects whether specific exons are included in the final transcript. For example, mutating the carboxy-terminal domain of Pol II, Pol II CTD, prevents the recruitment of the splicing regulatory protein, SRp20 (62). De la Mata et al. have shown in heptoma Hep3B, that transfection with mutant Pol II compared to wild-type impairs transcription of an alternatively spliced variant of their fibronectin extra domain I minigene. SRp20 overexpression is able to recue this transcript variant.

1.5.2 Alternative splicing and cancer

Alternative splicing is currently recognized as a major contributor to a variety of human diseases, including cancer. As mentioned above, regulatory elements exist throughout the gene and can be spatially quite far from the actual splice site. This makes previously disregarded synonymous mutations of greater interest because of their ability to deregulate splicing and
impact the pathology of a disease. Understanding the complex machinery, diversity of activators and repressors, and its multiple layers of regulation are critical for deciphering how splicing profiles are altered in a tumor cell. Furthermore, identifying and distinguishing different splice variant functions is proving to be an important component in understanding cellular deregulation in cancer.

1.6 ESTROGEN-RELATED ORPHAN NUCLEAR RECEPTOR β

1.6.1 Orphan nuclear receptors

Orphan nuclear receptors are a large subgroup within the nuclear receptor (NR) superfamily, comprising nearly half of all nuclear receptors. They are considered orphan because they have no known ligand to date. If a ligand is discovered, the orphan nuclear receptor is no longer categorized as an orphan, as was the case with PPAR, RXR, LXR, FXR, PXR/SXR, and CAR (63). Other than an unidentified ligand, orphans differ from classical NRs in a few ways. First, within their DNA-binding domains, they have a carboxy-terminal extension region (CTE). Classical NRs require dimerization for DNA binding, but the CTE allows orphans to bind DNA response elements as monomers (Fig 5) (64). Therefore, orphans have their own unique DNA response elements and functions, independent of classical NRs. Additionally, members of the ONR family have the smallest ligand binding pockets of all NRs (~100Å³) (63).

1.6.2 ERR structure

The estrogen-related orphan nuclear receptors (ERRs) have three family members, ERRα, β and γ. As their nomenclature suggests, they share significant sequence homology with
Figure 5. Dimerization partners and DNA response elements for classical nuclear receptors and orphan nuclear receptors (adapted from (64)).
the classical estrogen receptors (ERα and ERβ), namely in the DNA-binding domain, but do not bind estradiol (65). Of the ERRs, ERRβ and ERRγ are the most structurally similar (Fig 6). The crystal structure of the ERRs shows them in the active conformation with the ligand-binding pocket empty. Although they are constitutively active, synthetic small molecules and natural products are able to bind and stabilize the most active conformations of the ERRs. For example, the acyl hydrazone, DY131, has been shown to be a selective agonist of ERRβ and ERRγ, but does not effect ERRα, ERα or ERβ activity (Fig 7) (66).

1.6.3 ERRβ splice variants

The murine Esrrb gene produces a single confirmed mRNA encoding a protein of 433 amino acids, but in humans there exist two additional alternatively spliced forms of ESRRB with potentially distinct biological functions (Fig 8) (67,68). Short form ERRβ (ERRβsf) uses an intronic stop codon after exon 9 and is >90% homologous to mouse and rat ERRβ. ERRβ2 has an extended carboxyl-terminus encoded by exons 10, 11, and part of 12, while ERRβ-Δ10 splices exon 9 to exon 11 and includes all of exon 12. Due to a frame shift that occurs during alternative splicing, ERRβ-Δ10 and ERRβ2 each have a unique activation function 2 (AF2) region which is absent in ERRβsf (67).

ERRβsf was the first splice variant discovered and has been the most studied thus far, particularly in the area of stem cell research mentioned above. In terms of cancer, less is known for the role of any of the ERRβ splice variants. In a prostate cancer model looking exclusively at ERRβsf, Yu et al. demonstrated that ERRβsf can directly bind p21 and initiate a G1/S arrest, irrespective of p53 status (69). Furthermore, both the G1/S arrest and p21 induction were enhanced using the ERRβ agonist, DY131.
Figure 6. Sequence homology between ERR family members.
Figure 7. DY131, a synthetic, selective agonist of ERRβ and ERRγ. A, Chemical structure of DY131. B, Reporter gene assay comparing DY131 stimulation of fusion proteins containing the ERRs or ERs ligand-binding domains tethered to the Gal-4 DNA-binding domain in CV-1 cells (adapted from (66)).
Figure 8. ERRβ splice variants (adapted from (67)). A, ERRβ splice variant mRNA transcripts. B, ERRβ splice variant protein homology.
A separate study investigated whether ERRβsf and ERRβ2 could have unique functions, in part due to localization differences (67). Zhou et al. demonstrated that exogenously expressed ERRβsf is almost exclusively nuclear (>96% of interphase cells) while only ~30% of cells expressing exogenous ERRβ2 show nuclear localization. Furthermore, luciferase promoter-reporter assays have shown that exogenous ERRβ2 essentially lacks transcriptional activity when compared to ERRβsf (67). Current data suggests it is likely that ERRβ splice variants have different functions in cancer.

1.7 RESEARCH GOALS

As we start to decipher the different facets of cancer, it is clear that alternative splicing and splice variant function is becoming a critical field of research. Exploiting the role of novel splice variants in disease is a possible therapeutic strategy. In order to do so, it will be important to identify the new functions these splice variants take on through disease progression. Little is known about the endogenous role of ERRβ splice variants in cancer. Therefore, this study focuses on understanding the function of ERRβ splice variants in the context of glioblastoma multiforme (GBM) where two ERRβ splice variants, ERRβsf and ERRβ2, are expressed. Using the ERRβ synthetic agonist, DY131, as a tool to modulate endogenous ERRβ function, this research elucidates previously unknown roles for ERRβsf and ERRβ2 in cell cycle regulation and cell death.
CHAPTER 2
ERRβ SPLICE VARIANTS DIFFERENTIALLY REGULATE CELL CYCLE PROGRESSION
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2.1 Introduction

Orphan receptors comprise nearly half of all members of the nuclear receptor superfamily (70). Though these transcription factors apparently lack endogenous ligands, natural products, synthetic ligands or the binding of coregulatory proteins can modulate their constitutive activity. Estrogen-related receptors (ERRs) have broad structural similarity to canonical estrogen receptors alpha and beta (ERα, ERβ) though they cannot bind estrogen, and are well-established transcriptional regulators of mitochondrial biogenesis and function, including fatty acid oxidation, oxidative phosphorylation, and the tricarboxylic acid cycle (71).

ERRbeta (ERRβ) is required for proper placental formation in mice (72,73), and its conditional deletion in either the whole animal or specifically in neural progenitor cells increases lean body mass, energy expenditure, and feeding frequency by altering stress response signaling through the hypothalamic-pituitary-adrenal axis (74,75). ERRβ frame-shift and point mutations in the DNA- and ligand-binding domains are associated with autosomal recessive hearing loss (76).

The murine Esrrb gene produces a single confirmed mRNA encoding a protein of 433 amino acids, but in humans there exist two additional alternatively spliced forms of ESRRB with potentially distinct biological functions (67,68). Short form ERRβ (ERRβsf) uses an intronic stop codon after exon 9 and is >90% homologous to mouse and rat ERRβ. ERRβ2 has an extended carboxyl-terminus encoded by exons 10, 11, and part of 12, while ERRβ-Δ10 splices exon 9 to exon 11 and includes all of exon 12. Due to a frame shift that occurs during alternative splicing,
ERRβ-Δ10 and ERRβ2 each have a unique activation function 2 (AF2) region which is absent in ERRβsf (67).

Exogenous expression of ERRβsf has transcription-dependent tumor suppressor activities that engage the G1/S checkpoint in prostate cancer (69). However, the molecular function(s) of endogenous ERRβsf, or the ERRβ2 and ERRβ-Δ10 splice variants, in other tumor types remain unknown. Here, we evaluated a synthetic small molecule activator of ERRβ (DY131) (66) in cellular models of glioblastoma multiforme, and found that this agonist induces cell death in cancer, but not non-transformed, cell lines. Apoptotic cell death in response to DY131 requires mutation or loss/silencing of p53. In addition, p53 mutant T98G cells exhibit a robust G2/M arrest, a previously unreported finding. Using splice variant-selective shRNAs, we determined that silencing of ERRβ2, but not ERRβsf, abrogates DY131-induced G2/M arrest and cell death in p53-mutant T98G cells. In p53 wild type A172 cells, suppression of ERRbsf, but not ERRβ2, inhibits cell death and G1/S arrest. Lastly, we demonstrate that activation of ERRβsf can induce cellular senescence in a p53-independent manner. These results are the first to describe a function for endogenous ERRβ2 and reveal a novel interplay between ERRβ splice variants, which has broad implications for cell cycle control in glioblastoma and potentially other malignancies.

2.2 Materials and Methods

Cell Lines, Culturing Conditions, and Reagents—A172 and T98G cells were provided by Dr. Todd Waldman (Lombardi Comprehensive Cancer Center (LCCC), Georgetown University, Washington, DC). HEK293T cells were acquired from the LCCC Tissue Culture Shared Resource. HFF1 cells were obtained from Dr. Louis Weiner (LCCC, Georgetown University,
Washington, DC). RKO cells and variants were provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). All cells tested negative for \textit{Mycoplasma} spp. contamination, and were maintained in a humidified incubator with 95\% air: 5\% carbon dioxide. A172, T98G, and T98G stable cell lines were fingerprinted by the LCCC Tissue Culture Shared Resource to verify their authenticity using the standard nine STR loci and Y-specific amelogenin. A172, T98G and HEK293T cells were grown in IMEM supplemented with 10\% FBS. HFF1 cells were grown in high glucose Dulbecco's Modified Eagles Medium (DMEM; Thermo Scientific) supplemented with 15\% FBS.

G418 was purchased from the LCCC Tissue Culture Shared Resource and used at a final concentration of 1.2 mg/ml for A172- and T98G-shERR\(\beta\) stable cells. Puromycin was purchased from Life Technologies (Carlsbad, CA) and used at a final concentration of 2mg/ml for A172-shp53 stable cells. DY131 (Tocris Bioscience, Ellisville, MO) was dissolved in dimethyl sulfoxide (DMSO), stored as 10mM stocks at -20\(^{\circ}\)C, and used at the concentrations indicated. Hexadimethrine bromide (polybrene) was purchased from Sigma (St. Louis, MO).

\textit{Cell Cycle Analysis}—Cells were seeded at a density of 75,000-100,000 cells per well in 6-well plastic tissue culture dishes on day 0. The following day cells were treated with vehicle or drug. On day 2, Floating cells were collected. Adherent cells were trypsinized and added to the collected, floating cells. Cells were pelleted by centrifuging for 5min at 1,000 RPM. Media was aspirated and cells were washed once with cold 1x PBS and centrifuged again. PBS was aspirated and the cells were then fixed in 75\% ethanol. SubG1 (propidium iodide (PI) staining) and cell cycle (DNA content) analyses were performed by the LCCC Flow Cytometry and Cell Sorting Shared Resource.
**Cell Proliferation Assay**—Cells were seeded at a density of 1,000 cells per well in 5, 96-well plastic tissue culture dishes per cell line on day 0. On day 1, one plate was stained with crystal violet (untreated). The remaining plates were dosed with vehicle or DY at the specified concentrations. Plates were re-dosed every 72h and stained on days 3, 6, 10 and 14. For staining, plates were rinsed 1x with 1x PBS to remove excess cellular debris. After, 100ul of 0.5% crystal violet in 25% methanol was added to each well and incubated at 4C for 10min. The stain was then removed and the plate was rinsed 4-6x with diH2O to remove excess stain. The plates were left to air-dry overnight. On day 15, all plates were rehydrated with a 0.1M sodium citrate buffer solution in 50% ethanol and read at an absorbance of 550nm.

**Colony Formation Assay**—Cells were seeded at a density of 250 (A172) or 200 (T98G) cells per well in a 12-well plastic tissue culture dish on day 0. On day 1, cells were treated with the indicated doses of DY. The drug was removed on day 2, cells were washed 1x with 1x PBS before returning the cells to their normal culture media (in the absence of DY) for the remainder of the assay. Media was changed one time throughout the assay. Wells were stained on day 10 with 1ml crystal violet (as above) and left to dry overnight before counting colonies. Images were taken on a Nikon SMZ1500 fluorescence stereoscope at 0.375x magnification.

**Immunoblotting**—Cells were lysed in modified radioimmunoprecipitation assay (RIPA) buffer (77) supplemented with CompleteMini protease inhibitor and PhosSTOP phosphatase inhibitor tablets (Roche Applied Science, Penzburg, Germany). Polyacrylamide gel electrophoresis and protein transfer were performed as described previously (77,78). Membranes were blocked in 5%
nonfat dry milk buffer, unless otherwise noted, and incubated overnight at 4°C with primary antibodies for: PARP (1:1000), total histone H3 (1:1000), phospho-H3 serine 10 (1:1000) (all from Cell Signaling, Beverly, MA), p53 (1:1000, clone BP53-12, Millipore, Temecula, CA), p21 (1:300, Santa Cruz Biotechnology, Santa Cruz, CA), ERRβ (1:1000, clone H6707 (cl.07) and 1:500 clone H6705 (cl.05), R&D Systems manufactured by Perseus Proteomics, Tokyo), ERRγ (1:100, Abcam, Cambridge, MA). ERRγ purified protein (transcript variant 2) was purchased from Origene (Rockville, MD). As a loading control, all membranes were re-probed with β–actin primary antibody (1:10,000, Sigma) for ≥1 hour at room temperature. Horseradish peroxidase-conjugated secondary antibodies (1:5000, GE Healthcare Life Sciences, Pittsburgh, PA) and enhanced chemiluminescent detection HyGLO™ Quick Spray Chemiluminescent (Denville, South Plainfield, NJ) were used for detection as decribed in (78).

Annexin V Assay—On day 0, cells were seeded at a density of 100,000 cells per well in 6-well plastic tissue culture dishes. Cells were treated with DY on day 1 for 24h. Floating cells were collected. Adherent cells were trypsinized and added to the collected, floating cells. Cells were pelleted by centrifuging for 5min at 1,000 RPM. Media was aspirated and cells were washed once with 1x PBS and centrifuged again. PBS was aspirated and cells were washed once with 500ml binding buffer (BioLegend, San Diego, CA) and centrifuged. Binding buffer was aspirated and 5ml of Annexin V antibody conjugated with FITC (BioLegend, San Diego, CA) was added to the cell pellets and lightly vortexed. Samples were incubated for 15min at room temperature, in the dark, before adding 400ml of binding buffer. PI was added and levels of FITC and PI were measured by the LCCC Flow Cytometry and Cell Sorting Shared Resource.
**Lentiviral shRNA and stable cell lines**—Short hairpin RNA (shRNA) directed against human TP53 (pLKO-p53-shRNA-941) and the empty pLKO.1 vector were provided by Dr. Todd Waldman (79). shRNAs directed against ERRβ and the control scrambled insert in psiLv-mU6 were purchased from Genecopeia (Rockville, MD). The lentiviral helper plasmids for pLKO viral packaging, pHR’8.2ΔR and pCMV-VSV-G, were provided by Dr. Chunling Yi (LCCC, Georgetown University, Washington, DC). To prepare viral stocks, HEK293T cells were seeded at a density of 1.5 million cells per 100mm plastic tissue culture dish. Packaging cells were triply transfected using Lipofectamine LTX and Plus reagent (Life Technologies) and the following ratios of plasmids: 4µg pLKO-p53-shRNA-941 or empty pLKO.1, 3µg pHR’8.2ΔR and 2µg pCMV-VSV-G. For production of shERRβ-containing virus, cells were co-transfected with shRNA or scrambled control plasmids using the Lenti-Pac FIV Expression Packaging Kit (Genecopoeia) according to manufacturer’s instructions. Media was changed the next day. Supernatant was collected 48h post-transfection, centrifuged, aliquoted and stored at -80°C. Knockdown was assessed at the protein level by immunoblotting.

**Shp53:** CACCATCCACTACAACTACAT  
**shERRβ-1:** TGAGGACTACATCATGGAT  
**shERRβ-2:** TGCAGCACTTCTATAGCGT

**ERRβ expression constructs**—ERRβsf (murine ERRβ, >90% homology to human ERRβsf) was initially purchased from Addgene (Cambridge, MA; plasmid #40798) (80). The insert was amplified by PCR, purified using a GE Illustra GFX kit, digested with EcoRI and BamHI restriction enzymes (Promega, Madison, WI) and cloned into the recipient pSG5 vector that was
also digested with EcoRI and BamHI using standard molecular biology techniques. Proper insertion was confirmed by automated DNA sequencing (Genewiz, Germantown, MD) and this plasmid has been re-deposited at Addgene (#52188). The ERRβ2 and ERRβ-Δ10 splice variants were synthesized and cloned into pSG5 by Genewiz with codon optimization to confer resistance to shERRβ-1 and shERRβ-2, and have also been deposited at Addgene (#s 52186 and 52187, respectively).

Transfection of ERRβsf and ERRβ2—Cells were seeded at a density of 100,000 (T98G) or 150,000 (A172) cells per well in 6-well plastic tissue culture dishes on day 0. On d1, cells were transfected using either jetPRIME (T98G, Polyplus, Illkirch, FRA) or Lipofectamine LTX and Plus reagent (A172) according to manufacturer’s instructions. After 4h, media was change containing either DMSO or indicated concentrations of DY. Protein was harvested 24h post-DY treatment.

Senescence Assay—Cells were seeded at 15,000-20,000 cells per well in 12-well plastic tissue culture dishes on day 0. The following day cells were treated with vehicle or drug and then a senescence associated β-galactosidase staining kit (#9860, Cell Signaling) was used 24h post-treatment to detect cellular senescence. Images were taken on an Olympus IX-71 inverted epifluorescence microscope at 20x magnification in brightfield.

Image Analysis and Statistics—NIH Image J (http://rsbweb.nih.gov/ij/) was used to perform densitometry. Statistics were performed using GraphPad Prism software 5.0. Analyses used in this study include one-way ANOVA followed by Tukey’s multiple comparisons posttest or two-
way ANOVA followed by Bonferroni’s posttest. In all figures, data are presented as the mean ± standard deviation (SD). Statistical significance is defined by a $P$ value of $\leq 0.05$.

2.3 Results

*DY131 inhibits cellular proliferation, induces cell death and prevents colony formation in cancer cells, but not in non-transformed control cells* — Exogenous expression of ERRβsf inhibits the growth of prostate cancer cells (69), but the molecular function(s) of endogenous ERRβsf, or the other splice variants of this receptor, remain unknown. To address this, we used the acyl hydrazone DY131 (DY), a synthetic agonist of ERRβ (66), in a pair of glioblastoma multiforme (GBM) cell lines. We first exposed A712, T98G (GBM) and HFF1 cells (non-transformed human foreskin fibroblasts) to DY, then stained DNA with crystal violet at various time points for up to 14 days to determine whether DY affects cellular proliferation (Fig 9A). DY selectively impaired growth in both cancer cell lines, but not in HFF1 cells. To determine whether these results were due to cytotoxic (cell death) versus cytostatic (cell cycle arrest) effects, cells were treated with DY for 24h and we measured the fraction of cells that stained positive for fragmented DNA (subG1) (Fig 9B). DY induced cell death in the cancer lines, but not in HFF1 cells. Similar to our proliferation assay results, T98G cells were more sensitive to DY treatment at lower doses than A712 cells. We also tested whether DY could prevent colony formation in cancer cell lines (Fig 9C). DY significantly impaired A72 and T98G cells’ ability to form colonies. We further verified that these differences in DY-induced cell death were not attributable to variations in basal proliferation rates between the two cancer cell lines (Fig 9D).

DY is also an agonist for ERRgamma (ERRγ) (66), which is 77% identical to ERRβsf and whose exogenous expression can also inhibit the growth of prostate cancer cells (81). The
A. Figure 9. DY131 inhibits cellular proliferation, induces cell death and prevents colony formation in cancer cells, but not in non-transformed cells. A, A crystal violet assay staining the DNA (measured by absorption at 550nm) of A172, T98G, and HFF1 to show growth rates in the presence or absence of DY at indicated concentration.
Figure 9 (continued): B, Fraction of cells containing fragmented DNA (PI positive) 24h after DY treatment determined by flow cytometry (n=3, one-way ANOVA). C, Representative images and quantification of a colony formation assay. Cells were seeded on day 0, treated with indicated DY on day 1 before drug was washed out on day 2. Plates were stained and colonies counted on day 10 (n=4, one-way ANOVA). (*p<0.05 **p<0.01 ***p<0.001)
D. Crystal violet assay demonstrating basal cellular growth rates. E. Basal ERRβ and ERRγ protein expression. Lighter exposure films are included in ERRβ western blots to emphasize splice variant specificity of cl.07 (for ERRβ2) and cl.05 (ERRβsf) antibodies. Lanes labeled Δ10, β2, and SFB2 contain whole cell lysate from T98G cells transiently transfected with the indicated cDNA.
precise mechanism by which DY enhances the constitutive transcriptional activity of these orphan nuclear receptors is not known, though a related compound increases the overall stability of the ERRγ ligand-binding domain in thermal stability assays (82). We therefore examined basal protein expression of ERRβ and ERRγ in our cell lines (Fig 9E). Two commercially available antibodies from R&D Systems preferentially detect endogenous ERRβ2 and ERRβsf splice variants in A172 and T98G cells (cl.07 and cl.05, respectively). The cl.05 antibody can also detect the third splice variant (ERRβΔ10), but its endogenous expression is minimal in these cells. HFF1 cells express very low levels of the ERRβ splice variants. By contrast, ERRγ expression is robust in both the GBM and non-transformed cell lines.

*DY131 mediates cell cycle arrest*—Given the anti-proliferative effects of DY and the difference in p53 status between A172 (wild-type) and T98G (mutant) cells, we examined whether these effects were also accompanied by a cell cycle arrest. In A172 cells, we found DY induced a G1/S arrest after 24h (Fig 10A). Interestingly, the same treatment in T98G cells caused a G2/M arrest (Fig 10B). We then identified specific G1/S (p53 and p21) and G2/M (phospho-H3ser10) protein markers to confirm cell cycle arrest signaling in each cell line (Fig 10C). A172 cells, which arrest in G1/S, showed a corresponding induction of two major G1/S checkpoint regulators: p53 and its downstream target, p21. In T98G cells, we did not observe an induction of G1/S checkpoint mediators, but DY induced phosphorylation of histone H3 at serine 10, previously shown to be a specific phosphorylation site during prophase and important for chromatin condensation (83,84). These data suggest DY induces a cell cycle arrest specifically in mitosis in T98G cells.
Figure 10. DY131-mediated cell cycle arrest differs between p53 wild type and p53 mutant GBM cells. A, Fraction of p53 wild type A172 cells in G1/S and mean cell cycle profile after 24h DY treatment determined by flow cytometry (n=5, one-way ANOVA). B, Fraction of p53 mutant T98G cells in G2/M and mean cell cycle profile after 24h DY treatment determined by flow cytometry (n=5, one-way ANOVA). C, Protein expression for p53, p21 and phospho-H3 ser10 in A172 and T98G cells after 24h DY treatment. (*p<0.05**p<0.01***p<0.001)
Loss of p53 function promotes DY131 mediated apoptosis—To understand how DY causes cell death in A172 and T98G cells, we first performed an annexin V assay. After 24h of DY treatment, T98G cells showed a significant increase in annexin V and PI positive cells, whereas the A172 cells did not (Fig 11A). To confirm our annexin V data, we examined PARP cleavage and observed a similar result; DY induced PARP cleavage in T98G cells, but not A172 cells (Fig 11B). To test whether the magnitude of cell death and induction of apoptosis were dependent on loss of p53, we stably silenced p53 in A172 cells using lentiviral delivery of shRNA (79). We then measured the level of subG1 (Fig 11C) and PARP cleavage (Fig 11D) after DY treatment. p53 knockdown significantly increased the amount of DY-induced cell death at 5µM and 10µM, and caused PARP cleavage at 10µM. Because A172 and T98G cells have molecular differences other than p53 status, and PARP cleavage in A172-shp53 cells was not as robust as in parental T98G (p53 mut) cells, we used a second model to more directly test p53’s involvement in DY-mediated apoptosis: the RKO isogenic p53 null (p53−/−) system (85). First, we verified that DY protein targets (ERRβ and ERRγ) were detectably expressed in these cell lines (Fig 11E). RKO-p53−/− cells showed a significantly higher percentage of cell death than the RKO-p53+/+ parental cells when treated with DY (Fig 11F). Similar to our GBM p53 wild-type and p53 mutant pair, the RKO-p53−/− cells also showed a DY-mediated induction of PARP cleavage whereas the RKO-p53+/+ cells did not (Fig 11G). These two model systems support the conclusion that cells lacking wild type p53 (by mutation, silencing, or deletion) die by apoptosis when treated with DY.

ERRβ knockdown reverses DY-induced cell death and cell cycle arrest. DY is a synthetic agonist for both ERRβ and ERRγ that, thus far, we have demonstrated inhibits cellular proliferation and
Figure 11. Loss of wild-type p53 function promotes DY131-mediated apoptosis. A, Fold change of Annexin V and PI positive cells after 24h DY treatment relative to DMSO control (n=3, two-way ANOVA). B, Protein expression of PARP (full length (FL) and cleavage product (CP)) 24h after DY treatment. C, Percentage of A172-pLKO.1 and -shp53 stable cells in subG1 24h after DY treatment determined by flow cytometry (n=3, two-way ANOVA). D, Protein expression of PARP in A172-pLKO.1 and -shp53 stable cells. A lighter exposure of PARP is included to highlight the appearance of a cleavage product at 10mM DY treatment in shp53 cells. (*p<0.05**p<0.01***p<0.001)
Figure 11 (continued). E. Basal protein expression of ERRβ2 (cl.07) and ERRβsf (cl.05) in RKO isogenic mutants. Lanes labeled Δ10, β2, and SFβ2 contain whole cell lysate from T98G cells transiently transfected with the indicated cDNA. F. Fraction of RKO cells in subG1 after 24h DY treatment determined by flow Cytometry (n=4, two-way ANOVA). G. PARP protein expression in RKO isogenic mutants 24h post-DY treatment. (*p<0.05**p<0.01***p<0.001)
induces death specifically in cancer cells. Because only the cancer cell lines express readily detectable ERRβ protein, while cancer and non-transformed lines all express ERRγ (Fig 9E), we postulated that ERRβ was responsible for the DY-mediated cell death and cell cycle arrest phenotypes. To test this hypothesis, and verify DY specificity, we stably transduced T98G cells with lentiviral vectors containing two different shRNAs against ESRRB, or a scrambled control. Interestingly, each shRNA preferentially targeted a different ERRβ splice variant (ERRβsf was silenced by shERRβ-2 and ERRβ2 by shERRβ-1) (Fig 12A). In these modified T98G cells, knockdown of ERRβ2 significantly reduced cell death caused by DY treatment (Fig 12B). Knockdown of ERRβsf also showed a modest reduction in cell death, but only in the presence of 10 µM DY (Fig 12B). Strikingly, silencing of ERRβ2, but not ERRβsf, completely reversed the DY-mediated G2/M arrest in T98G cells (Fig 12C). These data are further supported by the reversal of protein signaling indicative of apoptosis (PARP cleavage) and G2/M arrest (phospho-H3 ser10) (Fig 12D). We also compared the basal growth rates of the T98G stable cells to parental T98G cells (Fig 12E) and saw no difference in proliferation, confirming these results were not due to a fundamental change in cellular proliferation resulting from stable infection. Altogether, these data support a role for ERRβ2 in regulation of the cell cycle in mitosis as well apoptotic cell death.

We used the same approach to silence ERRβ2 and ERRβsf in A172 cells. Stable knockdown of ERRβsf reduced the level of DY-induced cell death, whereas silencing of ERRβ2 did not (Figs 12F and G). Similarly, knockdown of ERRβsf reversed the G1/S arrest caused by DY (Fig 12H) and reduced the induction of p53 and p21 (Fig 12I). Finally, we examined the basal growth rates of the A172 stable cell lines and again found no significant difference (Fig 12J). These data demonstrate that ERRβsf activates a G1/S checkpoint through p53 and/or p21.
Figure 12. ERRβ knockdown reverses DY131-mediated cell death and cell cycle arrest. A, ERRβsf (cl.05) and ERRβ2 (cl.07) protein expression in T98G shERRβ stable cells. Lanes labeled Δ10, β2, and SFβ2 contain whole cell lysate from T98G cells transiently transfected with the indicated cDNA. B, Percentage of T98G shERRβ stable cells in subG1 after 24h DY treatment determined by flow cytometry (n=3, two-way ANOVA).
(*p<0.05**p<0.01***p<0.001)
Figure 12 (continued). C, Percentage of T98G shERRβ stables cells in G2/M and mean cell cycle profiles after 24h DY treatment determined by flow cytometry (n=3, two-way ANOVA). (*p<0.05 **p<0.01 ***p<0.001)
Figure 12 (continued). D, PARP and phospho-H3$^{\text{ser}10}$ protein expression in control cells compared to shERRβ2 cells after 24h DY treatment. E, Crystal violet assay staining total DNA (measured by absorption at 550nm) to determine basal growth rates of T98G parental and shERRβ stable cells.
Figure 12 (continued). F. Percentage of A172 shERRβ stable cells in subG1 after 24h DY treatment determined by flow cytometry (n=3, two-way ANOVA). G. Western blot of A172 shERRβ stable cell lines using ERRβ-cl. 07 antibody to demonstrate ERRβ2 knockdown by shERRβ-1. Lanes labeled Δ10, β2, and SFβ2 contain whole cell lysate from T98G cells transiently transfected with the indicated cDNA. (*p<0.05**p<0.01***p<0.001)
Figure 12 (continued). H, Fraction of A172 shERRβ cells in G1/S 24h and mean cell cycle profiles after DY treatment determined by flow cytometry (n=3, one-way ANOVA). (*p<0.05**p<0.01***p<0.001)
I. Protein expression of p53, p21 and ERRβsf in A172 control cells compared to A172 shERRβsf stable cells. J. Crystal violet assay staining total DNA (measured by absorption at 550nm) to determine basal growth rates of A172 parental and shERRβ stable cells.

**Figure 12 (continued).** I, Protein expression of p53, p21 and ERRβsf in A172 control cells compared to A172 shERRβsf stable cells. J, Crystal violet assay staining total DNA (measured by absorption at 550nm) to determine basal growth rates of A172 parental and shERRβ stable cells.
ERRβ splice variant, not p53 status, determines phase of DY131-mediated cell cycle arrest. To determine the role of p53 in DY-induced cell cycle arrest, we examined the A172-pLKO.1 and –shp53 stable cells. We observed a significant reduction in the level of G1/S arrest in the A172-shp53 cells relative to the controls (Fig 13A). However, while the magnitude of the arrest was less than in control cells, A172-shp53 cells still underwent a dose-dependent G1/S arrest in response to DY. Furthermore, knockdown of p53 protein did not completely abolish the p21 induced by DY (Fig 13A). Together, these data imply that p53 is not the sole contributor to DY-mediated G1/S arrest, and that ERRβsf can activate p21 independent of p53. This latter finding is consistent with (69), who show in a prostate cancer model that exogenous expression of ERRβsf causes G1/S arrest via direct transcriptional upregulation of p21 and DY amplifies this arrest and p21 induction. Furthermore, parental and p53+/− isogenic variant RKO cells both arrested in G2/M (Fig 13B). Interestingly, we observed an induction of p53 and p21 in the RKO-p53+/− cells, even though there was not a corresponding G1/S arrest. In both RKO cell lines, we saw phosphorylation of histone H3 at serine 10, consistent with the observed cell cycle arrest in G2/M (Fig 13B).

Additional data strongly suggest ERRβ splice variants have different functions in cell cycle regulation, independent of p53. In T98G cells, silencing ERRβ2, but not ERRbsf, completely reversed the DY-mediated G2/M arrest (Fig 12C, light gray bars).

Interestingly, this G2/M arrest reversal was accompanied by a G1/S arrest and p21 induction, not previously observed in T98G parental cells (Fig 13C). These data imply a potential dominant inhibitory role for ERRβ2, where in the absence of this splice
Figure 13. ERRβ isoform determines phase of cell cycle arrest, not p53 status. A, Percentage of A172-pLKO.1 and -shp53 stable cells in G1/S and mean cell cycle profiles (determined by flow cytometry) (n=3, two way ANOVA) after 24h DY treatment and corresponding protein expression of p53 and p21. (*p<0.05**p<0.01***p<0.001)
Figure 13 (continued). B, Percentage of RKO isogenic mutants in G2/M (n=4, one-way ANOVA) after 24h treatment and mean cell cycle profiles (determined by flow cytometry) and corresponding protein expression of p53, p21 and phospho-H3 ser10. (*p<0.05**p<0.01***p<0.001)
Figure 13 (continued). C, Fraction of T98G shERRβ stable cells in G1/S and mean cell cycle profiles (determined by flow cytometry) (n=3, one-way ANOVA) and corresponding p21 protein expression. (*p<0.05**p<0.01***p<0.001)
variant, ERRβsf is now able to induce p21 and initiate a G1/S arrest. Importantly, this occurs in the presence of mutant p53, again consistent with the ability of ERRbsf to regulate p21 directly (69).

Moreover, silencing of ERRβsf in T98G cells led to an enhanced G2/M arrest (Fig 12C, speckled bars). We speculate ERRβsf and ERRβ2 interact – physically and/or functionally – in such a way that removal of ERRbsf from the system permits amplified signaling through ERRβ2, leading to a stronger arrest in G2/M after treatment with DY. Our RKO cell cycle and protein signaling data further suggest an interplay between ERRβ splice variants. Despite induction of p21 by DY (likely through activation of ERRβsf), the RKO-p53<sup>+/+</sup> cells still arrest in G2/M (Fig 13B). This could be explained by a non-functional p21 in RKO-p53<sup>+/+</sup> cells, but this seems unlikely given others have shown p21’s ability to induce a G1/S arrest in this cell line (86). We hypothesize ERRb splice variant interaction is a critical component for DY-mediated cell cycle regulation.

To confirm the specific function of ERRβ2 and ERRβsf in DY-mediated G2/M and G1/S arrest, respectively, we transiently transfected ERRβ-silenced cells with plasmids encoding shRNA-resistant versions of the appropriate splice variant. Exogenous expression of ERRβ2 in T98G-shERRβ2 cells rescued both the apoptotic response and G2/M arrest phenotype in DY-treated cells (Fig 13D). Similarly, exogenous expression of ERRβsf in A172-shERRβsf cells restored p53 and p21 induction by DY (Fig 13E). Taken together, these data illustrate a dynamic interplay between the cell cycle regulatory functions of these ERRβ splice variants, and demonstrate for the first time that ERRβ2 specifically drives G2/M arrest.
Figure 13 (continued). D, Protein expression of PARP, ERRβ2 and phospho-H3<sup>ser10</sup> in T98G-shERRβ2 cells transfected with the shRNA-resistant ERRβ2 plasmid (28h) and treated with DY (24h). E, Protein expression of p53, p21 and ERRβsf in A172-shERRβsf cells transfected with the shRNA-resistant ERRβsf plasmid (28h) and treated with DY (24h). (*p<0.05 **p<0.01 ***p<0.001)
ERRβsf induces cellular senescence independent of p53. Thus far, we and others (69) have demonstrated that ERRβsf can cause G1/S arrest and induce p21. Given the critical role for p21 in cellular senescence (87), either downstream of p53 or in a p53-independent manner, we asked whether DY could also induce senescence associated β-galactosidase in A172 cells (Fig 14A).

We detected a dose-dependent relationship for cellular senescence caused by DY. To establish that this phenotype required ERRβsf expression, we performed the same assay in our A172-shERRβsf stable cells, and found that knockdown of ERRβsf markedly reduced the level of senescence caused by DY (Fig 14B).

To determine whether p53 plays any role in the observed senescent phenotype, we first measured senescence in RKO-p53+/+ and RKO-p53−/− cells treated with DY (Fig 14C). Despite clear p53 and p21 induction in RKO-p53+/+ cells following DY exposure (Fig 13B), these cells did not undergo senescence. There was also no evidence of senescence in T98G cells treated with DY (Fig 14D). Furthermore, when we assayed our A172-shp53 cells, we saw no reduction in DY-induced cellular senescence (Fig 14E), despite residual p21 induction (Fig 13A).

Collectively, these data demonstrate that DY-mediated cellular senescence requires ERRβsf, and imply that p53 and p21 are neither necessary nor sufficient for the senescent phenotype.
2.4 Discussion

ERRβ is an orphan nuclear receptor with established roles in mitochondrial function and metabolic control, placental development in mice, and a specific genetic deafness disorder in humans. Exogenous overexpression studies with one of the three alternatively spliced forms of ERRβ have suggested a role for this receptor in growth inhibition and cell cycle arrest in prostate cancer, but the molecular function(s) of endogenous ERRβ in this and other tumor types remain unknown. Here, using a synthetic, small molecule activator of ERRβ in glioblastoma multiforme cell line models, we demonstrate novel cellular functions for the ERRβ2 and ERRβsf splice variants that have broad implications for cell death and cell cycle control in glioblastoma and perhaps other malignancies.

We used stable transduction of two different ESRRB-targeted shRNAs to selectively silence ERRβ2 and ERRβsf in A172 and T98G cells. Surprisingly, the target sequences for both shRNAs are present in both splice variants. Local structure of target mRNA is known to contribute to the efficiency of RNA interference. shERRβ-2 targets a sequence in the ESRRB gene close to the end of exon 9, where use of an intronic stop codon yields ERRβsf but conventional splicing produces ERRβ2. Using Mfold (88), we demonstrate that the predicted secondary structure in this region differs notably between the two splice variants (Fig 15), with the shERRβ-2 target site taking a more favored loop structure (89,90) in ERRβsf (where silencing is observed) vs. a less favored stem-like structure in ERRβ2 (where silencing is not observed). Structural differences between splice variants do not explain ERRβ2-selective silencing by shERRβ-1, however, since the predicted secondary structures for the target sequence of this shRNA are the same in both. Additional factors such as tertiary structure, pre-mRNA
**Figure 15.** Mfold prediction for ERRβsf (A) and ERRβ2 (B) mRNA structure. First predicted secondary structure using the minimum free energy (MFE) algorithm at [mfold.rit.albany.edu](http://mfold.rit.albany.edu) is shown for each splice variant. shERRβ-1 and shERRβ-2 sequences are highlighted blue.
binding proteins, and/or loading of shRNAs into the RISC complex may contribute to this selectivity (91).

Although the tumor suppressor p53 is widely considered to positively regulate apoptosis, particularly in cancer where restoration of p53 function is an ongoing therapeutic challenge (92), DY only induces apoptosis in the absence of wild type p53. We comprehensively show that loss of wild type p53 function is associated with (or required) for DY-mediated apoptosis, using three different models (p53 mutant T98G cells, RKO cells with isogenic deletion of p53, and A172 cells silenced for p53 using shRNA). A seminal paper by Polyak et al. established that p53’s ability to induce apoptosis in colon cancer cells is determined by two independent mechanisms: p21-dependent cell cycle arrest, which blunts p53-induced apoptosis, and the engagement of pro-death signaling partners that directly contribute to apoptosis (93). Our data most clearly support the first mechanism – that p21-dependent cell cycle arrest is cytoprotective – because in each of our three models, p21 induction is absent (T98G), lost (RKO-p53−/−), or reduced (A172-shp53) when apoptosis occurs. Future studies will establish the direct, pro-apoptotic targets of DY.

While DY-mediated apoptosis requires loss/silencing or mutation of p53, our data suggest that activation of ERRβsf induces cellular senescence that is independent of p53. Cell enlargement and senescence-associated β-galactosidase staining are only induced in G1/S-arresting A172 cells, not in G2/M-arresting RKO cells (which are also p53 wild type; Fig 6A and C). In addition, silencing of ERRβsf in A172 cells markedly reduces the senescent phenotype, while silencing of p53 does not (Fig 14B and D). Although p53 and its upstream regulator ARF are often considered the predominant inducers of cellular senescence, a number of p53-independent mechanisms can contribute to this tumor suppressive mechanism (87), and the
CDKN2A/B locus that encodes ARF shows homozygous deletion in A172 cells (94). Direct regulation of p21 by ERRβsf could be responsible for DY-mediated senescence, given that many ARF/p53-independent senescence mechanisms still rely on p21, and that p21 upregulation in response to DY is prevented by ERRβsf silencing (Fig 12G) vs. modestly inhibited by knockdown of p53 (Fig 13A) in A172 cells. However, RKO-p53+/+ cells, which do not senesce, still show induction of p21 in response to DY. This could suggest that i. pro-senescence signaling downstream of p21 is fundamentally different in RKO cells, or ii. ERRβsf drives a p21-independent senescence pathway.

A key novel finding of our study is that ERRβ2 can mediate G2/M arrest in response to DY. Silencing of ERRβ2, but not ERRβsf, in T98G cells blocks G2/M arrest, and this is rescued by exogenous expression of shRNA-resistant ERRβ2 (Figs 12C and 13D). To our knowledge, this is the first demonstrated function for endogenous ERRβ2. Luciferase promoter-reporter assays have shown that exogenous ERRβ2 essentially lacks transcriptional activity when compared to ERRβsf. Also, while exogenously expressed ERRβsf is almost exclusively nuclear (>96% of interphase cells), only ~30% of cells expressing exogenous ERRβ2 show nuclear localization of the receptor (67). Given these data, and that transcription is generally repressed during mitosis, it is likely that transcription-independent functions of ERRβ2 are responsible for the G2/M arrest phenotype. Another orphan nuclear receptor with a unique function in mitosis is NR5A1 (steroidogenic factor-1, SF1), silencing of which promotes centrosome duplication and chromosomal instability through a transcription-independent mechanism involving DNA-PK (95). SF1 has a centrosome localization signal. ERRβ2 does not, but sequence analysis using Scansite (96) shows that its unique AF2 domain includes a proline-rich sequence with significant homology to the Src homology 3 (SH3) domain-binding site for cortactin. Interestingly,
cortactin localizes to the spindle poles during mitosis, where its presence is required for proper centrosome separation by serving as an anchor for actin filaments (97). The ability of DY to stimulate phosphorylation of histone H3 at serine 10 is supportive of a role for ERRβ2 in early mitotic arrest, since this site gets phosphorylated during prophase, is important for chromatin condensation, and dephosphorylation occurs during anaphase (83,84). Future studies will focus on the potential mechanisms by which activated ERRβ2 perturbs G2/M cell cycle progression.

Alternative splicing represents an important source of functional diversity for the proteome (54). In many cases, splice variants have opposing roles (e.g. pro-survival BclXL vs. pro-apoptotic BclXS) (98) and/or serve as dominant-negative inhibitors of each other (e.g. Ets-1 p51 and p27) (99). Our data suggest that ERRβ2 is dominant-inhibitory for ERRβsf. In T98G cells, silencing of ERRβ2 suppresses DY-mediated G2/M arrest and apoptosis (Fig 12A-C), which now permits G1/S arrest and p21 induction (Fig 13C). Several important questions remain: what cellular factors dictate the choice between production of ERRβsf and ERRβ2?; when they are co-expressed, how is splice variant phenotypic dominance determined in relation to cell cycle arrest?; and at the molecular level, how does ERRβ2 repress ERRβsf? These will be important considerations in the design of future studies to address the novel functional interaction between the ERRβ2 and ERRβsf splice variants.
CHAPTER 3

CONCLUSIONS AND FUTURE DIRECTIONS
CONCLUSIONS AND FUTURE DIRECTIONS

In this study, we identify previously unknown roles for both ERRβsf and ERRβ2 in cell cycle regulation, apoptosis and/or senescence. We show that activated ERRβsf causes a G1/S arrest through induction of p53 and p21. Using shRNA targeted against ERRβsf we were able to reverse the G1/S arrest, partially reduce the p53 induction and more completely reverse the p21 induction. Furthermore, re-expressing ERRβsf in shERRβsf stable cells rescued the G1/S signaling cascade through an increase in p53 and p21 expression. Finally, we show activated ERRβsf can induce cellular senescence and we are able to reverse this phenotype in shERRβsf stable cells.

For the first time, we illustrate a role for ERRβ2 in mitosis, specifically at the prophase/metaphase interchange. We show ERRβ2 activity leads to a G2/M arrest in the cell cycle. Phosphorylation of histone H3 at serine 10 indicates this arrest occurs during the very late stages of prophase, immediately before metaphase during mitosis. Silencing of ERRβ2 using shRNA completely reverses this arrest and pH3Ser10 expression. We are able to rescue the mitotic signaling through exogenous expression of ERRβ2 in shERRβ2 stable cells.

Interestingly, our data also suggests ERRβ2 may be a dominant negative splice variant for ERRβsf. When we silence ERRβ2 in a cell line where ERRβ2 was previously shown to cause mitotic arrest, we observe an induction of p21 and a corresponding G1/S arrest. This implies ERRβsf is active in the absence of ERRβ2 and can activate target genes such as p21 (Fig 16). It still remains unclear as to how and why which splice variant is dominant in a cell. One possibility is the complement of cofactors and the role they may play in splice variant activity.
Figure 16. Signaling model summarizing ERRβ splice variant function and interaction in cell cycle regulation
Finally, we demonstrate that either splice variant is able to induce cellular toxicity in a p53-independent manner. Our data strongly implies the interaction between ERRβsf and p21 protects the cell from apoptosis whereas ERRβ2 initiates an apoptotic response. The mechanism behind ERRβ2-mediated apoptosis remains unclear and future studies using caspase inhibitors are needed to determine whether the intrinsic and/or extrinsic pathways are involved.

DY131 has demonstrated efficacy, albeit in a limited capacity in our in vitro models, in cancer-specific cell death. Interestingly, DY sensitivity was enhanced in the absence of functional p53. Disruption of wild-type p53 function plays a central role in the development and progression many cancer types. Across all cancer types, p53 is the most commonly mutated or lost gene. Therefore, DY131’s selective potency in the absence of wild-type p53 has the ability to reach a profound number of cancer patients. In vivo modeling and toxicity testing would be the next step in determining DY131 safety in humans.

In this study, we have not addressed the possibility that ERRγ may be playing a compensatory role in cell cycle regulation following ERRβ silencing. For example, it is possible that knockdown of ERRβ2 leads to the activation of ERRγ resulting in the observed G1/S arrest. However, given the current literature demonstrating the distinct role for ERRβsf in G1/S transition, it is more plausible ERRβsf is responsible for the arrest in the absence of ERRβ2. Even so, double knockdown experiments of ERRβ2 and ERRγ would be needed to conclusively exclude the role of ERRγ.

This research has generated many more questions that still need answers. For future studies, understanding the molecular mechanisms behind the ERRβ2-mediated mitotic arrest seems the most fascinating, particularly given the lack of research on ERRβ2 function. Our preliminary data in T98G as well as another p53-mutant breast cancer cell line, MDA-MB-231,
suggests ERRβ2 may exert its effects on mitosis in a transcriptionally independent manner. Using stably transfected GFP-H2B T98G and MDA-MB-231 cells, we arrested cells in G2/M using a nocodozole block. We then released the cells directly into DY131 and monitored the cells in real time progress through mitosis using the same methods as described by Ringer et al. (100) (Fig 17). We show control cells successfully complete mitosis in less than one hour, as expected. In the presence of DY131, the cells not only remain in metaphase for hours without ever completing cell division (monitored up to five hours), their metaphase plate appears triangulated with multiple spindles. Mitotic catastrophe is a tumor suppressive mechanism that arrests cells experiencing a defective M phase, ultimately leading to apoptosis or other forms of cell death (101). Given the timescale of our live imaging experiment and DY exposure, it is likely ERRβ2 is orchestrating mitotic catastrophe independent of a transcriptional target. Because of the disorganization of the metaphase plate, in addition to phosphorylation of histone H3, it is possible ERRβ2 may be targeting an aurora kinase. Aurora kinases (A and B) each have distinct functions during mitosis to ensure proper spindle formation, chromosome alignment and chromosome segregation. Specifically, Aurora A coordinates the function of the plus-end-directed motor protein CenpE (102). CenpE transports polar chromosomes from the spindle pole to the mitotic equator to form the metaphase plate. The timing of these events is tightly regulated to ensure proper division and avoid misalignment or aneuploidy. Therefore, the mitotic defects and concurrent mitotic arrest could be due to premature Aurora kinase activation by ERRβ2.

The role of ERRβ2 in human cancers is almost completely unknown. This data suggests it may be a cancer-specific ERRβ splice variant. If so, ERRβ2 could be a novel target for
Figure 17. DY131 causes abnormal metaphase. MDA-MB-231 cells stably expressing GFP-H2B were synchronized at the G2/M boundary by 40 ng/ml nocodazole for 16h before release into DMSO vehicle or DY131 and live imaging on a Nikon TE300 spinning disk confocal microscope. Representative images are shown for normal mitosis (top row) and the defect(s) caused by DY131 (bottom row), including chromatin structures suggestive of tripolar spindles.
therapy. This project contributes to deciphering the basic function of ERRβ splice variants as a first step in determining whether these are potential therapeutic targets for cancer in the future.
APPENDIX I

ERK/MAPK REGULATES ERRγ EXPRESSION, TRANSCRIPTIONAL ACTIVITY,
AND RECEPTOR-MEDIATED TAMOXIFEN RESISTANCE IN ESTROGEN
RECEPTOR+ BREAST CANCER
ERK/MAPK REGULATES ERRγ EXPRESSION, TRANSCRIPTIONAL ACTIVITY, AND RECEPTOR-MEDIATED TAMOXIFEN RESISTANCE IN ER+ BREAST CANCER

Al.1 Introduction

Worldwide, breast cancer is the most common cancer in women, with an estimated 1.38 million new cases diagnosed per year (103), and ~70% of breast cancers are estrogen receptor alpha-positive (ER+). ER+ breast cancer can be successfully treated with selective estrogen receptor modulators (SERMs) such as Tamoxifen (TAM) (104), and ER is one of only two robust, reproducible biomarkers that are routinely used to make breast cancer treatment decisions in the clinic (105). However, the development of TAM resistance is a pervasive problem that affects nearly half of all women with ER+ breast cancer who are treated with TAM (106-108). Typically, it is not loss or mutation of ER that causes resistance, but changes in proliferative and/or survival pathways in an ER+ breast tumor cell that override the inhibitory effects of TAM. These frequently include alterations in receptor tyrosine kinases, cell cycle regulatory proteins, and mediators of apoptosis.

Distinct from hormone-regulated nuclear receptors such as ER, 25 members of this protein superfamily lack an identified ligand and are thus designated orphan nuclear receptors (70). Orphan nuclear receptors display constitutive transcriptional activity and have been implicated in numerous developmental and disease processes, including breast cancer (109). A trio of estrogen-related receptors (ERRα, β, and γ) are well established transcriptional regulators of mitochondrial biogenesis and function, including fatty acid oxidation, oxidative phosphorylation, and the tricarboxylic acid cycle (71,110) in organs and tissues with high energy
requirements, such as the heart and liver. Multiple studies have now shown that the ERRs alter metabolism and oncogene expression in breast and other cancer cells in a way that promotes growth and proliferation (111,112). In non-transformed mammary epithelial cells, upregulation of endogenous ERR\(\gamma\) after detachment from the extracellular matrix contributes to metabolic reprogramming and, ultimately, the development of resistance to anoikis (113).

As their name implies, ERRs have broad structural similarity to classical ER, but being orphan nuclear receptors they have no (known) endogenous ligand and do not bind estrogen. The third member of this family, ERR\(\gamma\) (ESRRG, NR3B3), is preferentially expressed in ER+ breast cancer (114). Endogenous ERR\(\gamma\) is upregulated during the acquisition of TAM resistance by ER+ invasive lobular breast cancer cells, and exogenous expression of ERR\(\gamma\) in this breast cancer type is sufficient to induce TAM resistance (78). ERR\(\gamma\) mRNA is also significantly increased in pre-treatment tumor samples from women with ER+ breast cancer who ultimately relapsed following TAM treatment (109). More recently, nuclear expression of ERR\(\gamma\) protein has been shown to correlate with lymph node-positive status in a small cohort of breast cancer patients (115), and gene-level amplification of ERR\(\gamma\) is significantly enriched in lymph node metastases vs. the primary breast tumor (116).

The goal of the current study is to better understand how ERR\(\gamma\) expression and activity are regulated, and how this regulation contributes to the TAM resistant phenotype in ER+ breast cancer. We show herein that i) modulation of ERK activity directly affects ERR\(\gamma\) protein levels, ii) Serines 57, 81, and 219 are required for ERK-mediated enhancement of ERR\(\gamma\) protein, and iii) mutation of these sites abrogates receptor-mediated TAM resistance and reduces transcriptional activity.
AI.2 Materials and Methods

Cell Lines, Culturing Conditions, and Reagents—ER-positive, Tamoxifen-responsive MCF7 cells were originally obtained from Dr. Marvin Rich (Karmanos Cancer Institute, Detroit, MI). The ER-positive, Tamoxifen-resistant variant of MCF7 (MCF7/RR cells) was a kind gift of Dr. W. B. Butler (Indiana University of Pennsylvania, Indiana, PA) (117). ER-positive, Tamoxifen-responsive SUM44 cells have been described previously (78). All cells tested negative for Mycoplasma spp. contamination, and were maintained in a humidified incubator with 95% air: 5% carbon dioxide. MCF7 and MCF7/RR cells were cultured in modified improved minimal essential medium (IMEM; Life Technologies, Grand Island, NY) with phenol red (10 mg/L) supplemented with 5% fetal bovine serum (FBS). SUM44 cells were cultured in serum-free Ham’s F12 medium (1.25 mg/L phenol red) with insulin, hydrocortisone, and other supplements (SFIH) as described previously (78,118).

4-hydroxytamoxifen (4HT; Sigma, St. Louis, MO) was dissolved in 200-proof ethanol, stored as a 10 mM stock at -20°C, and used at the concentrations indicated. The MEK inhibitor U0126, JNK inhibitor SP600125 and p38 inhibitor SB203580 (Tocris Bioscience, Ellisville, MO) were dissolved in dimethyl sulfoxide (DMSO), stored as 10 and 50mM stocks (respectively) at -20°C, and used at the concentrations indicated. Poly-L-lysine was purchased from Sigma. Recombinant human epidermal growth factor (EGF) was purchased from PeproTech (Rocky Hill, NJ) and used at the concentration indicated.

Expression Constructs and Reporter Plasmids—An ORF cDNA clone for human ERRγ (AB020639.1) was purchased from GeneCopoeia (Rockville, MD). Wild type, HA-tagged murine ERRγ (pSG5-HA-ERR3, 100% protein sequence identity to human ERRγ transcript
variant 1) has been described previously (78,119). The serine-to-alanine variants (S$_{45}$A and S$_{57,81,219}$A) were generated using the QuikChange Lightning site-directed mutagenesis kit (Stratagene, La Jolla, CA), confirmed by automated DNA sequencing (GENEWIZ, South Plainfield, NJ), and have been deposited at Addgene (Cambridge, MA; plasmid #s 37849 and 37850, respectively). Amino acid numbers correspond to transcript variant 1. Plasmids encoding constitutively active MEK (pBabe-puro-MEK-DD, (120)) and wild type, HA-tagged ERK2 (pCDNA-HA-ERK2 WT, (121)) were obtained from Addgene (plasmid #s 15268 and 8974, respectively).

The estrogen response element (ERE)-containing promoter reporter construct (3xERE-luciferase) has been described previously (78,122). To generate the estrogen-related response element (ERRE)-containing reporter (3xERRE-luciferase, (123)) and the hybrid ERRE/ERE-responsive reporter (3xERRE/ERE-luciferase, (124)), oligonucleotides were synthesized (IDT, Coralville, IA), annealed, and cloned into KpnI/BglII-digested pGL3-Promoter vector (Promega, Madison, WI) using standard techniques. Oligonucleotide sequences are as follows:

ERRE forward:
5’…CCGGACCTCAAGGTCACGTTCCGACCTCAAGGTCACTGTTCCGGACCTCAAGGTTCGGACCCTCAAGGT
CAGGATCCA…3’

ERRE reverse:
5’…gatctGGATCCTGACCTTGAGGTCGAACGTGACCTTGAGAACGTGACCTTGAGG
GTCCGggtac…3’

ERRE/ERE forward:
5’…CCGGACCTCAAGGTCACCTTGACCTCGTTCGGACCTCAAGGTCACCTTGACCTC
TTCGGACCTCAAGGTCACCTTGACCTGGATCCA…3’
ERRE/ERE reverse:

5’…gatctGGATCCAGGTCAGGTGACCTGAGGTCCGAAAAGGTTGACCTTGA
GAACGAGGTCAGGTGACCTTGAAGGTCCGgtac…3’

**Bold** indicates consensus ERRE sequences, **underlined italics** indicate consensus ERE sequences, and small letter sequences highlight KpnI and BglII sites. Proper annealing and insertion were confirmed by automated DNA sequencing (GENEWIZ), and plasmids have been deposited at Addgene (plasmid #s 37851 and 37852, respectively).

**Clinical Data**—The KM Plotter tool (http://kmplot.com/analysis/) (125) was used to evaluate ERRγ mRNA expression (Affymetrix ProbeID 207981_s_at) in publicly available breast cancer gene expression data from 65 patients selected by the following parameters: overall survival (OS), upper vs. lower tertile of ESRRG expression, ER-positive tumors (including those for which ER+ status is extrapolated from gene expression data), Tamoxifen as only form of endocrine therapy, and any chemotherapy.

**Reverse Transcription PCR (RT-PCR)**—RNA was extracted from subconfluent monolayers of exponentially growing cultures using the RNEasy Mini kit (Qiagen, Valencia, CA). One microgram of total RNA was DNase treated and reverse transcribed using Super Script II and other reagents from Life Technologies. Quantitative RT-PCR was performed for individual cDNA samples (1:5 dilution) using TaqMan Gene Expression Assays for ESRRG and RPLP0 as described previously (78). Standard (non-quantitative) RT-PCR was performed on 400 ng of cDNA or 800 pg of the human ERRγ ORF cDNA clone with primers designed to amplify ESRRG or RPLP0 using TaqSelect DNA polymerase from Lucigen (Middleton, WI) under the
following PCR conditions: 94°C for 2 min; 35 cycles of 94°C for 30 sec, 54°C for 30 sec, and 72°C for 1 min 24 sec; final extension of 72°C for 10 min; 4°C hold.

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<td>ESRG</td>
<td>GGAGGTCGGCAGAAGTACAA</td>
<td>GCTTCGCCCATCCAATGATAAC</td>
<td>241 bp</td>
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<tr>
<td>RPLP0</td>
<td>ACCATTGAAATCCTGAGTGA</td>
<td>AATGCAGAGTTTCTCTGTG</td>
<td>187 bp</td>
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*Transient Transfection and Immunoblotting*—Cells were seeded on 6-well, 12-well, or 100 mm plastic tissue culture dishes one day prior to transfection with the indicated expression constructs using Lipofectamine 2000 or Lipofectamine LTX (Life Technologies), or JetPrime (VWR, Radnor, PA) according to the manufacturer’s instructions. For transfections using Lipofectamine 2000, wells were pre-coated with poly-L-lysine. Transfection complexes were removed (and, where indicated, 4HT or kinase inhibitors were added) at 4-6 hours post-transfection. For the growth factor stimulation experiment, 4-6 hours post-transfection the cells were washed twice in sterile PBS and cultured in low-serum (0.5% FBS) conditions overnight (~20 hours) before treatment with EGF in the presence or absence of U0126 for 2 hours. For both transfected and non-transfected cells, wells and dishes were lysed in modified radioimmunoprecipitation assay (RIPA) buffer (77) supplemented with CompleteMini protease inhibitor and PhosSTOP phosphatase inhibitor tablets (Roche Applied Science, Penzburg, Germany). Polyacrylamide gel electrophoresis and protein transfer were performed as described previously (77,78). Nitrocellulose membranes blocked in either 5% nonfat dry milk or 7.5% bovine serum albumin
(BSA) in Tris-buffered saline plus Tween (TBST) for ≥1 hour were incubated overnight at 4°C with primary antibodies for: phosphorylated Erk1/2 (1:1000), total Erk1/2 (1:1000), total MEK (1:1000), phosphorylated JNK (1:5000), total JNK (1:500), phosphorylated p38 (1:1000), total p38 (1:1000), phosphorylated Rb Ser780 (1:1000), total Rb (1:1000) (all from Cell Signaling, Beverly, MA); ERRγ (1:100, ab82319 from Abcam, Cambridge, MA); p21 (1:300, sc-756), p27 (1:500, sc-528) from Santa Cruz Biotechnology, Dallas, TX; or the HA epitope tag (1:500, HA.11 clone 16B12, Covance, Princeton, NJ). For ERRγ detection, 25 ng of purified protein corresponding to human ERRγ transcript variant 2 (Origene, Rockville, MD) was run alongside 67 µg whole cell lysates. As a loading control, all membranes were re-probed with β–actin primary antibody (1:5000-1:10,000, Sigma) for ≥1 hour at room temperature (78). Horseradish peroxidase-conjugated secondary antibodies (1:5000) and enhanced chemiluminescent detection were performed as described previously (78).

**FACS Analysis of Bromodeoxyuridine (BrdU) Incorporation**—MCF7 cells were seeded in poly-L-lysine-coated 6-well plastic tissue culture plates at a density of 2.5 x 10⁵ cells per well, respectively, one day prior to transfection with 4 µg HA-ERR3, the S₅₇,₈₁,₂₁₉A variant, or empty vector (pSG5) using Lipofectamine 2000. Four to 6 hours post-transfection, transfection complexes were removed and cells were treated with 1 µM 4HT or ethanol vehicle. 48 hours later, BrdU was added to a final concentration of 10 µM for an additional 18-20 hours. Cells were fixed and stained using the APC (allopheocyanin) BrdU Flow Kit with 7-AAD (7-amino-actinomycin D; BD Pharmingen, San Jose, CA) according to the manufacturer’s instructions with one modification: during incubation with the APC-conjugated anti-BrdU antibody, cells were co-stained with AlexaFluor488-conjugated anti-HA antibody (Covance) at 1:50-1:100.
Fluorescence-activated cell sorting (FACS) was performed on a BD FACSARia instrument. For wild type- and mutant-transfected cells, data are presented for only HA-positive (*i.e.* AlexaFluor488-stained) cells; for empty vector-transfected cells, data are presented for all sorted cells.

*Promoter-Reporter Luciferase Assays*—MCF7 and SUM44 cells were seeded in poly-L-lysine-coated 24- and 12-well plastic tissue culture plates at $7.5 \times 10^4$ and $2.0 \times 10^5$ cells per well, respectively. The following day, cells were co-transfected with 500 or 1000 ng HA-ERR3, the S$_{57,81,219}$A variant, or empty vector (pSG5), 290 or 580 ng 3xERE-, 3xERRE-, or 3xERRE/ERE-luciferase, and 10 or 20 ng pRL-SV40-Renilla (internal control), respectively. Transfection complexes were removed and media were replaced 4-6 hours post-transfection. Twenty-four (MCF7) and 48 (SUM44) hours post-transfection, cells were lysed and analyzed for dual-luciferase activity as described previously (78).

*Image Analysis and Statistics*—NIH Image J (http://rsbweb.nih.gov/ij/) was used to perform densitometry. All statistical analyses were performed using GraphPad Prism 5.0c for Mac (La Jolla, CA), with the exception of the hazard ratio and logrank p value in Fig. 1A, which were generated by the KM Plotter tool. All data are presented as the mean ± standard deviation (SD), and statistical significance is defined as $p \leq 0.05$. qRT-PCR, BrdU incorporation, and promoter-reporter luciferase assays were analyzed by t test or one-way analysis of variance (ANOVA) with *post-hoc* Tukey’s or Dunnet’s multiple comparison tests.

**AI.3 Results**
ERRγ mRNA (ESRRG) is increased in pre-treatment tumor samples from women with ER+ breast cancer who relapse within 5 years of TAM treatment (109,126). Using the KM plotter tool (125) to test whether there is an association between ERRγ and other clinical parameters in additional patient populations with longer follow-up time, we found that high expression of ESRRG (upper vs. lower tertile) is significantly associated with worse overall survival in ER+ breast cancer patients who received TAM as their only endocrine therapy (Fig 17A, hazard ratio 2.44, logrank p = 0.035). MCF7/RR cells are a TAM-resistant variant of MCF7 (117) that depend on heightened signal transduction through networks regulated by nuclear factor kappa B (NFκB) (127) and glucose-regulated protein 78 (GRP78) (128) for maintenance of the resistance phenotype. By quantitative RT-PCR, expression of ERRγ (Fig. 17B) is increased in resistant MCF7/RR cells vs. sensitive, parental MCF7s. However, MCF7 cells have a mean cycle threshold (C\text{T}) greater than 35, indicative of very low expression outside the optimal range of TaqMan gene expression assays; the mean C\text{T} for MCF7/RR cells is 33. We subsequently performed non-quantitative RT-PCR for ESRRG in independent samples of MCF7 and MCF7/RR cells alongside a human ERRγ ORF cDNA clone (Fig. 17C). While ESRRG mRNA is detectable in both cell lines, the signal intensity observed in ~400 ng cDNA is 40-50% less than that obtained from 800 pg of plasmid. By Western blot, MCF7 and MCF7/RR cells have undetectable ERRγ protein in 67 µg of whole cell lysate, while 25 ng of purified ERRγ protein is observed (Fig. 17D). These data show that MCF7 and MCF7/RR cells express very low levels of receptor mRNA, and that endogenous ERRγ protein is not readily detected in these cells by the available commercial antibodies.

We therefore adapted an exogenous expression model (MCF7 cells transiently transfected with a hemagglutinin (HA)-tagged ERRγ (78,119)) to determine the mechanism(s) by which this
Figure 17. ERRγ expression in ER+ breast tumors and breast cancer cells. A, Expression of ESRRG in ER+, TAM-treated breast tumors is associated with worse overall survival. HR = hazard ratio calculated by (125). B, Relative expression of ESRRG normalized to RPLP0 in MCF7 and MCF7/RR cells by quantitative RT-PCR. Mean cycle threshold (C_T) values for parental (MCF7) cells are shown. Bars, n=3 replicates from a representative assay performed independently twice. Error, standard deviation (SD). **p≤0.01 for t test. C, Expression of ESRRG and RPLP0 in MCF7 and MCF7/RR cells by non-quantitative RT-PCR. Upper and lower arrowheads identify ESRRG and RPLP0 amplicons, respectively. Plasmid denotes ERRγ ORF cDNA clone. D, Expression of ERRγ protein in MCF7 and MCF7/RR cells by Western blot analysis. *denotes a non-specific band detected by the ERRγ antibody. Purified protein denotes human ERRγ transcript variant 2. β–actin = loading control.
orphan nuclear receptor, when expressed, might modulate the TAM-resistant phenotype. Post-translational modifications such as phosphorylation play essential roles in the regulation of many proteins, including nuclear receptors.

At least 8 different phosphorylation sites have been shown to regulate expression or activity of classical (ligand-regulated) ER (129), and a number of these have clinical significance in women with breast cancer who are treated with TAM (106,130). In the absence of identified ligand(s), the activity of orphan receptors is thought to be particularly sensitive to regulation by phosphorylation (131-135). ERK hyperactivation has been associated with TAM resistance *in vivo* and *in vitro* (136,137), and inhibition of its upstream regulator MEK improves the anti-tumor activity of the steroidal antiestrogen Fulvestrant in ER-positive ovarian cancer (138). Therefore, we tested whether the activity of ERK or the two other major members of this kinase family (JNK and p38) directly affect exogenous ERRγ in MCF7 cells (**Fig. 18A**, left panels). The minimal consensus sequence required for phosphorylation of a substrate by any member of the MAPK family is the dipeptide motif S/T-P (139), and ERRγ contains 4 serines (no threonines) that meet these criteria: amino acids 45, 57, 81, and 219. Pharmacological inhibition of pERK by U0126 strongly reduces exogenous ERRγ (HA) levels, but inhibitors of p38 (SB203580) or JNK (SP600125) do not. Furthermore, co-transfection with a mutant, constitutively active form of MEK (MEKDD, (140)) increases pERK and enhances ERRγ (HA) levels (**Fig. 18B**), as does co-transfection with wild type ERK2 (**Fig 18C**). Stimulating MCF7 cells with EGF also increases pERK and enhances exogenous ERRγ (HA), and these effects are blocked by co-treatment with U0126 (**Fig 18D**). Finally, pharmacological inhibition of pERK by U0126 inhibits exogenous ERRγ (HA)
Figure 18. Effect of MEK and ERK on ERRγ protein levels. A, Inhibition of ERK, but not p38 or JNK, reduces exogenous ERRγ expression. MCF7 cells were transiently transfected with the pSG5 empty vector or HA-ERRγ, then treated with DMSO vehicle, 5 μM U0126 (MEK inhibitor), 25 μM SB203580 (p38 inhibitor), or 10 μM SP600125 (JNK inhibitor) for 24 hours prior to lysis and Western blot analysis. Left panels show ERRγ (HA) levels, phosphorylated ERK (pERK), and total ERK from a representative experiment repeated at least twice. Right panels show total and phosphorylated p38 and JNK (p-p38 and pJNK, respectively) from the
same experiment. β–actin = loading control. B, Constitutively active, mutant MEK enhances ERRγ protein levels. MCF7 cells were transiently co-transfected with HA-ERRγ and either MEKDD or additional pSG5 empty vector. *denotes the transfected MEKDD construct. β–actin = loading control. C, Exogenous, wild type ERK2 enhances ERRγ protein levels. MCF7 cells were transiently co-transfected with HA-ERRγ and either MEKDD, wild type HA-tagged ERK2, or additional pSG5 empty vector. *denotes the transfected MEKDD construct. The arrowhead and ^ denote transfected HA-ERRγ and HA-ERK2, respectively. β–actin = loading control. D, EGF-mediated enhancement of ERRγ protein levels is reversed by concomitant ERK inhibition. MCF7 cells were transiently transected with HA-ERRγ, then cultured in low-serum conditions for 20 hours before treatment with DMSO vehicle, 25 ng/ml EGF, or 25 ng/ml EGF plus 5 µM U0126 for 2 hours. β–actin = loading control. E, Inhibition of ERK reduces exogenous ERRγ expression in a second ER+ breast cancer cell line. SUM44 cells were transiently transfected with the pSG5 empty vector or HA-ERRγ, then treated with DMSO vehicle or 5 µM U0126 for 22 hours. β–actin = loading control.
expression in a second ER+ breast cancer cell line, SUM44 (Fig 18E). These data strongly suggest that ERRγ can be positively regulated by ERK.

The putative ERK phosphorylation sites in ERRγ are either located in the N-terminal activation function 1 (AF1) region of the protein (amino acids 45, 57, 81), or in the hinge region downstream of the DNA binding domain (amino acid 219). Tremblay et al. (141) have shown that ERRγ and its family member ERRα are regulated by a phosphorylation-dependent SUMOylation motif (PDSM). Phosphorylation at ERRγ S45 directs SUMOylation at K40, leading to repression of ERRγ transcriptional activity, and when this serine is mutated to alanine (S45A), ERRγ expression and transcriptional activity is enhanced. Therefore, we generated two different variants of ERRγ by site-directed mutagenesis: S45A (part of the PDSM), or S57,81,219A (unknown function). In contrast to wild type and S45A ERRγ, levels of the S57,81,219A variant are decreased by 70% compared to that of wild type ERRγ (Fig. 19A). To determine whether these 3 Serine residues are required for the MEK/ERK-mediated increase in ERRγ levels, wild type or S57,81,219A ERRγ was co-transfected with MEKDD (Fig. 19B). Consistent with data presented in Fig. 18B, activated MEK increases wild type ERRγ by ~3-fold. However, MEKDD is unable to enhance levels of the triple serine mutant. Similarly, treatment with U0126 reduces wild type ERRγ (HA) levels by 70% (consistent with Fig. 18A), but has no further effect on S57,81,219A ERRγ (Fig. 19C). Serines 57, 81, and 219 therefore appear to be required for regulation of ERRγ protein levels by ERK, and their mutation to alanine reduces basal receptor expression.

We next compared S57,81,219A ERRγ to the wild type receptor for its ability to induce TAM resistance. We first used 5-bromo-2’-deoxyuridine (BrdU) incorporation analyzed by fluorescence activated cell sorting (FACS) to measure changes in DNA synthesis (S phase) following 4HT treatment in MCF7 cells transiently transfected with empty vector (control), wild
Figure 19. Contribution of serines 57, 81, and 219 to ERRγ protein levels. A, Concomitant serine-to-alanine mutation at residues 57, 81, and 219 reduces basal HA-ERRγ levels. B, MEKDD fails to increase protein levels of S<sub>57,81,219</sub>A HA-ERRγ. C, Erk inhibition does not reduce S<sub>57,81,219</sub>A HA-ERRγ. MCF7 cells were transiently transfected and treated with 5 µM U0126 or DMSO vehicle for 24 hours where indicated (C) prior to lysis and Western blot analysis. β–actin = loading control. Densitometric values for the ratio of HA:β–actin are normalized to the level of wild-type receptor in the absence of treatment (1.0). Data are from representative experiments that were performed independently at least 3 times.
Figure 20. Effect of S\textsubscript{57,81,219}A mutation on Tamoxifen response. A, Inhibition of BrdU incorporation by 4HT is reversed by wild type but not S\textsubscript{57,81,219}A HA-ERR\textgamma. MCF7 cells were transiently transfected as shown, treated with ethanol vehicle or 1 µM 4HT for 48 hours, then incubated with BrdU for an additional 18-20 hours before fixation and staining for HA and BrdU. Dashed line denotes BrdU incorporation in vehicle-treated cells (set to 1.0). Points, n=3 independent assays. Error, SD. *p<0.05 for post hoc Dunnet’s test following one-way ANOVA for pSG5 vs. wild type HA-ERR\textgamma; n.s. denotes no statistical significance between pSG5 and S\textsubscript{57,81,219}A mutant HA-ERR\textgamma. For transfections with wild type or S\textsubscript{57,81,219}A ERR\textgamma, data are from HA-positive, FACS-sorted cells only. For transfections with the empty vector pSG5 control, data are from all cells in the population. B, 4HT-mediated induction of cell cycle inhibitors p21 and p27 is reversed by wild type but not S\textsubscript{57,81,219}A HA-ERR\textgamma, and the phosphorylation state of Rb is differentially affected by wild type vs. mutant receptor. MCF7 cells were transiently transfected as shown, then treated with ethanol vehicle or 2.5 µM 4HT for 21 hours prior to lysis and Western blot analysis. β–actin = loading control. Densitometric values for the ratio of the indicated proteins to β–actin in 4HT-treated conditions are normalized to the level of their expression in the absence of treatment (1.0) for each transfected construct; for pRb Ser780, the ratio of phosphorylated:total signal (which was then normalized to β–actin) is shown. Data are from a representative experiment that was performed independently 3 times.
type, or mutant ERRγ (Fig. 20A). As expected, 4HT reduces DNA synthesis by 50% in control (pSG5-transfected) cells. Wild type ERRγ confers significant resistance to 4HT (*p<0.05), but S₅₇,₈₁,₂₁₉A ERRγ does not. We then tested whether 4HT-mediated induction of the cyclin-dependent kinase (CDK) inhibitors p21 and p27, markers of G0/G1 arrest that are essential for TAM-mediated growth inhibition (142,143), are altered by exogenous ERRγ. Similar to its effect on ER (144), 4HT increases the expression of both wild type and S₅₇,₈₁,₂₁₉A ERRγ (Fig. 20B). However, the ~1.5-fold and 1.3-fold induction of p21 and p27, respectively, by 4HT in empty vector transfected cells is reduced or blocked by exogenous expression of wild type, but not mutant, ERRγ. We also measured total and phosphorylated levels of the retinoblastoma tumor suppressor (Rb), a target of active cyclin D1/CDK complexes and another indicator of G1 cell cycle progression. The role of Rb in TAM response and resistance is somewhat contradictory. Some studies report a reduction in pRb in responsive cells following TAM treatment, while others show that loss or downregulation of total Rb is associated with TAM resistance in cell culture models, xenografts, and premenopausal women with ER+ breast cancer (145,146). In vehicle-treated conditions, we observe a strong induction of total and pRb by wild type, but not S₅₇,₈₁,₂₁₉A, ERRγ. When treated with 4HT, the ratio of pRb to total Rb in wild type ERRγ-expressing cells is increased ~2-fold vs. vehicle treatment, and this is driven by a robust decrease in total Rb. In the presence of S₅₇,₈₁,₂₁₉A, ERRγ, pRb remains essentially constant but total Rb is increased in the presence of 4HT. Together, these data show that S₅₇,₈₁,₂₁₉A ERRγ is impaired in its ability to promote TAM resistance, and suggest that this may be due (at least in part) to altered regulation of cell cycle progression by mutant vs. wild type receptor.

ERRγ directly regulates transcription by binding to EREs or ERREs. Deblois et al. identified a hybrid ERRE/ERE element as the major binding site for the family member ERRα in
breast cancer (124). Because $S_{57,81,219}$ ERRγ does not induce TAM resistance, we tested whether this mutant has impaired transcriptional activity at all 3 response elements. In MCF7 cells, activity of mutant $S_{57,81,219}$ ERRγ is significantly reduced by ~30% vs. wild type ERRγ on the ERRE (Fig. 21A) and ERE (Fig. 21B). For the first time, we show that ERRγ can also stimulate transcription from the ERRE/ERE (Fig. 21C). However, activity of the $S_{57,81,219}$ mutant ERRγ at this hybrid element is decreased vs. wild type receptor by <10%. In contrast, the $S_{57,81,219}$ mutant ERRγ shows a 30-40% reduction in transcriptional activity at all 3 response elements in a different ER+ breast cancer cell line (SUM44) (Fig. 21D-F). These data demonstrate that ERK-mediated stabilization of ERRγ positively regulates receptor transcriptional function, and suggest that this is most relevant to ERRE- and ERE-driven activity.

A1.4 Discussion

In this study, we have shown that ERRγ protein levels are enhanced or stabilized by active ERK, mapped this activity to 3 Serine residues, and demonstrated that impairment of ERRγ phosphorylation at these sites reduces receptor-mediated TAM resistance and transcriptional activity in ER+ breast cancer cells. We propose that ERK-mediated phosphorylation of ERRγ is a key determinant of TAM resistance in ER+ breast cancer cells where this receptor is expressed and drives the resistant phenotype. To our knowledge this is the first demonstration of direct, functional consequences of phospho-regulation of a member of the ERR family. Ariazi et al. initially showed that ERRα transcriptional activity in ER+ breast cancer cells is enhanced by HER2 endogenous amplification (BT474) or exogenous expression (MCF7), and that pharmacological inhibition of AKT or MAPK reduces this activity (131). They also provide evidence, via in vitro kinase assays using GST-tagged ERRα constructs, that
Figure 21. Effect of $S_{57,81,219}$A mutation on ERRγ transcriptional activity. MCF7 and SUM44 cells were transiently co-transfected with pSG5 empty vector, wild type HA-ERRγ, or $S_{57,81,219}$A HA-ERRγ plus the ERRE- (A, D), ERE- (B, E), or ERRE/ERE-driven promoter-reporter luciferase construct (C, F) and the Renilla internal control for 24 (MCF7) or 48 hours (SUM44) prior to lysis and luciferase assay. Bars, luciferase:Renilla ratio of n=3 replicate wells from a representative assay performed 3 times independently. Error, SD. ***p ≤ 0.001 for one-way ANOVA with post hoc Tukey’s tests.
multiple receptor sites (particularly in the carboxy-terminus) can be phosphorylated by AKT and MAPK. However, Chang et al. reported that in SKBR3 (a HER2-amplified, ER- breast cancer cell line), expression of endogenous ERRα target genes is repressed by AKT, but not MAPK, inhibitors through regulation of the co-activator PGC1β (147). Moreover, they state that mapping and mutation of the proposed phosphorylation sites in ERRα has no effect on receptor transcriptional activity, which is in direct contrast to our finding that mutation of 3 ERK consensus sites in ERRγ significantly impairs transcriptional activity and receptor-mediated TAM resistance. That ERRα and ERRγ, despite their high sequence similarity and overlapping target genes, have differential functions in breast cancer is an idea that has gained considerable traction recently (111,148), and one that our future studies will address, particularly with respect to ERE- and ERRE-containing endogenous target gene selection (see below).

We were surprised by the apparent specificity of ERK for positive regulation of ERRγ in ER+ breast cancer cells. All three members of the MAPK family (ERK, JNK, p38) can phosphorylate the same S-P core motif, but our data show that only pharmacological inhibition of ERK reduces ERRγ protein. It should be noted that under these experimental conditions, p38 and JNK are expressed but their activation (phosphorylation) is minimal (Fig 18A, right panels). We therefore cannot rule out the possibility that in other contexts, ERRγ may have the capacity to be regulated by these other members of the MAPK family.

It is not yet clear how inhibition of ERK, or the S57,81,219A ERRγ mutation, ultimately leads to a decrease in receptor levels. One reasonable explanation is a change in proteasomal-mediated degradation of the receptor such that phosphorylation of serines 57, 81, and/or 219 by ERK slows or prevents ubiquitination and degradation of ERRγ. Our data showing that a brief, 2 hour stimulation with EGF is sufficient to enhance ERRγ (HA) expression would be consistent
with this. Similar to what we observe here, MEK/ERK-mediated stabilization of the GLI2 oncoprotein results in reduced ubiquitination of GLI2 that requires intact GSK3β phosphorylation sites (149). Parkin is the only E3 ubiquitin ligase that has so far been shown to ubiquitinate ERRγ (and other members of the ERR family) (150), but knowledge of whether/how parkin is impacted by ERK signaling in breast cancer is limited. In neurons parkin and MAPKs do act in opposition to regulate microtubule depolymerization (151), and in several breast cancer cell lines parkin has been reported to bind microtubules and stabilize their interaction with paclitaxel, leading to enhanced sensitivity to this chemotherapeutic drug (152). In MCF7 cells, exogenous parkin expression also independently attenuates cell proliferation by causing a G1 arrest (153). Future studies will determine whether ERK-dependent regulation of ERRγ requires the Parkin and ubiquitin/proteasome pathway.

A reduction in S\_57,81,219A mutant ERRγ protein levels, and its attendant failure to induce TAM resistance or promote cell cycle progression in MCF7 cells, is not perfectly correlated with impaired transcriptional activity. S\_57,81,219A mutant ERRγ is significantly less active at ERRE and ERE sites. However, Figure 21C shows that activity of the S\_57,81,219A mutant at the hybrid ERRE/ERE element is surprisingly near wild type in MCF7 cells, but reduced by 30% in SUM44 cells (Fig. 21F). Because these divergent results were obtained using identical, plasmid-borne heterologous promoter constructs (3 tandem ERRE/ERE sequences functioning as enhancers of the SV40 core promoter) under similar experimental conditions, we hypothesize that this context-dependent difference in mutant ERRγ activity could be due to a difference in either the repertoire of co-regulatory proteins, or the expression of ERα, in MCF7 vs. SUM44 cells. The latter possibility is interesting in light of what is known about the interplay between family member ERRα and ERα at these hybrid response elements. Using serial ChIP assays Deblois et
al. showed that in MCF7 cells, ERRα and ERα cannot simultaneously occupy these hybrid sites, and reduction of ERα by siRNA enriched ERRα binding to these sequences in the promoter regions of *FAM100A* and *ENO1* (124). We previously reported that SUM44 cells have high basal expression of ERα (78), which represents 3-fold enrichment in mRNA and protein levels vs. MCF7 cells (p<0.001, data not shown). This might mean that where competition with ERα is limited (*i.e.* in MCF7 cells), S$_{57,81,219}$A mutant ERRγ is more readily recruited to ERRE/ERE sites. However, S$_{57,81,219}$A mutant ERRγ is still unable to fully induce TAM resistance in MCF7 cells and shows compromised activity at ERE inverted repeats and the ERRE half site in these cells. This implies that phosphorylated, wild type ERRγ may preferentially activate ERE- and ERRE-regulated target genes to promote the TAM-resistant phenotype.
REFERENCES

Res. Tamoxifen Resistance in Novel Models of Invasive Lobular Breast Cancer


dependent on the local structure of mRNA at the targeted region. 


