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Estrogen-related receptor β (ERRβ) – renaissance receptor or receptor renaissance?

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Abbreviations: 4HT, 4-hydroxytamoxifen; **AIB1,** amplified in breast cancer 1; **AS,** alternative splicing; **BCP ALL,** B-cell precursor acute lymphoblastic leukemia; **BPA,** Bisphenol A; **BLAST,** basic local alignment search tool; **Cdc25A,** cell division cycle 25 homolog A; **cDNA,** complementary DNA; **CDKN1A,** cyclin-dependent kinase inhibitor 1A; **ChIP-seq,** chromatin immunoprecipitation and sequencing; **Crh,** corticotrophin releasing hormone; **Crh2,** corticotrophin releasing hormone 2; **CTE,** carboxyl-terminal extension; **DBD,** DNA-binding domain; **DES,** diethylstilbestrol; **DFNB,** nonsyndromic deafness, autosomal recessive; **dpc,** days post coitum; **Dub3,** deubiquitinating enzyme 3; **EC,** embryonal carcinoma; **ERα,** estrogen receptor α; **ERE,** estrogen response element; **ERRs,** estrogen-related receptors; **ERRβ,** estrogen-related receptor β; **ERRE,** estrogen-related response element; **ES,** embryonic stem; **ESC,** embryonic stem cell; **EST,** expressed sequence tag; **FRAP,** fluorescence recovery after photobleaching; **FRET,** fluorescence resonance energy transfer; **FTZ-F1,** fushi tarazu factor; **GBM,** glioblastoma; **GDOC,** Georgetown Database of Cancer; **GEO,** gene expression omnibus; **GR,** glucocorticoid receptor; **GRE,** glucocorticoid response element; **GST,** glutathione S-transferase; **HPA,** hypothalamic-pituitary axis; **IC50,** 50% inhibitory concentration; **iPSC,** induced pluripotent stem cell; **LBD,** ligand-binding domain; **LOH,** loss of heterozygosity; **MPM2,** mitotic protein monoclonal #2; **NCOA3,** nuclear receptor coactivator 3; **NLS,** nuclear localization sequence; **NPY,** neuropeptide Y; **Nrf2,** NF-E2 Related Factor 2; **OMIM,** Online Mendelian Inheritance in Man; **ONRs,** orphan nuclear receptors; **P,** postnatal; **PAX5,** paired box 5; **PGC,** primordial germ cells; **REMBRANDT,** Repository for Molecular Brain Neoplasia Data; **RID,** receptor interacting domain**; RIP140,** receptor interacting protein 140; **SAGE,** serial analysis of gene expression; **Shh,** Sonic hedgehog; **shRNA,** small hairpin RNA; **Smo,** Smoothened; **Sp1,** specificity protein 1; **SRC2,** steroid receptor coactivator-2; **TCGA,** The Cancer Genome Atlas; **TRα,** thyroid hormone receptor α; **TREpal,** palindromic thyroid hormone response element**; TSC,** trophoblast stem cell; **UTR,** untranslated region; **YFP,** yellow fluorescent protein

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Estrogen-related receptors (ERRs) are founding members of the orphan nuclear receptor (ONR) subgroup of the nuclear receptor superfamily. Twenty-seven years of study have yet to identify cognate ligands for the ERRs, though they have firmly placed ERRα (ESRRA) and ERRγ (ESRRG) at the intersection of cellular metabolism and oncogenesis. The pace of discovery for novel functions of ERRβ (ESRRB), however, has until recently been somewhat slower than that of its family members. ERRβ has also been largely ignored in summaries and perspectives of the ONR literature. Here, we provide an overview of established and emerging knowledge of ERRβ in mouse, man, and other species, highlighting unique aspects of ERRβ biology that set it apart from the other two estrogen-related receptors, with a focus on the impact of alternative splicing on the structure and function of this receptor.

Introduction

Gene discovery and orphan designation

A gene encoding estrogen receptor β (ERRβ), initially named hERR2 and subsequently designated NR3B2 or ESRRB, was first reported in 1988 [Giguère et al., 1988]. Giguère et al. screened a λgt10 human testis cDNA library with the DNA-binding domain of estrogen receptor α (ERα), isolated a sequence with only partial similarity, then screened that against a human adult heart cDNA library to isolate hERR2 (GenBank ID X51417.1). ERRα (initially designated hERR1) was isolated in parallel from a human fetal kidney cDNA library. The identified hERR2 open reading frame is 433 amino acids in length and contains broad sequence similarity to the DNA-binding domain (DBD) and ligand-binding domain (LBD) of ERα. Northern blot analysis showed that despite widespread expression of hERR1, hERR2 is

restricted to a single 4.8 kb transcript in rat heart, kidney, prostate, testis, and tissues of the central nervous system (including the hypothalamus), with human placenta and prostate testing negative for expression [Giguère et al., 1988]. Preliminary steroid binding studies suggested that hERR2 cannot bind androgens or estrogens, making it a founding member of the orphan nuclear receptor (ONR) subgroup.

Constitutive transcriptional activity of hERR2 was subsequently reported by two groups, who showed that replacement of either the progesterone receptor (PR) or glucocorticoid receptor (GR) LBD with amino acids 173-433 [Lydon et al., 1992] or 168-433 [Xie et al., 1999] of hERR2, respectively, drives ligand-independent activation of these chimeric receptors. Xie et al. also showed that intact hERR2 binds to the estrogen response element (ERE) and a palindromic thyroid hormone response element (TREpal), but not the glucocorticoid response element (GRE), and that constitutive activation of hERR2 is enhanced by co-transfection of p160 family coactivator proteins. Like ERα and other nuclear receptors, hERR2 can also indirectly modulate target gene transcription by coopting specificity protein 1 (Sp1) sites, including those in thyroid hormone receptor α (TRα) and cyclin-dependent kinase inhibitor 1A (CDKN1A, p21) [Castet et al., 2006], and appears to be a more potent activator of Sp1-driven transcription than either ERRα or ERRγ. By contrast, hERR2 represses GR-mediated transcriptional activity at GREs without altering the ability of the GR to bind DNA [Trapp and Holsboer, 1996] and is itself transcriptionally repressed by another ONR, DAX1 [Suzuki et al., 2003].

The mouse ortholog, mERR-2 (GenBank ID S82458.1), was cloned from undifferentiated embryonal carcinoma (EC) and embryonic stem (ES) cells [Pettersson et al., 1996]. A single 4.3 kb transcript (predicted open reading frame = 433 amino acids) is readily detected by Northern blot in undifferentiated murine F9 EC and ES cells, though only weakly expressed in adult mouse kidney and heart tissues, and retinoic acid-induced differentiation of F9s strongly suppresses mERR-2 expression. Functional studies with the mERR-2 cDNA in gel-shift assays show that, like hERR2, this receptor binds to EREs, but not direct repeats bound by retinoid X receptor heterodimers, and that hERR2 homodimerizes in solution and on EREs in a manner requiring functional heat shock protein 90 (hsp90) [Pettersson et al., 1996]. The murine and human genes were subsequently mapped to chromosomes 12 and 14q24.3, respectively [Sladek et al., 1997].

Table 1 summarizes the GenBank identifiers for human, mouse, and rat ESRRB.

A model for understanding ONR/DNA interactions

A distinctive feature of ONRs is the ability of many to bind as monomers to DNA sequences resembling half of the canonical steroid receptor inverted repeats, but with a 5' extension (termed extended half-sites, e.g. [Ikeda et al., 1993; Ueda et al., 1992]. In the related Drosophila melanogaster fushi tarazu factor (FTZ-F1) receptor, this capability had been attributed to a carboxyl-terminal extension (CTE) of the DBD [Ueda et al., 1992]. Wright and colleagues solved nuclear magnetic resonance (NMR) solution structures of the DBD of hERR2 (amino acids 96-194) in complex with DNA to formally prove that the CTE (amino acids 169-194) is required for hERR2 binding to what is now known as the estrogen-related response element (ERRE, sequence TCAAGGTCA), and that this is accomplished through insertion of the T- and A-boxes, also called an AT hook, into the minor groove of DNA (Protein Data Bank ID # 1LO1) [Gearhart et al., 2003; Sem et al., 1997]. CTE/DNA minor groove interactions involving the TCA sequence are essential for recognition by the hERR2 CTE, since synthetic polyamides (pyrrole-imidazole oligomers) that occlude it prevent DNA binding by hERR2 [Gearhart et al., 2005]. These detailed studies established hERR2 as a prototypical model for monomeric ONR/DNA interactions. It should be noted, however, that for other ERR family members, DNA binding more commonly occurs in a homodimeric fashion [Takacs et al., 2013; Vanacker et al., 1999], with deviance at the cytosine within the AT hook driving a preference for monomeric vs. dimeric binding of ERRα [Barry et al., 2006].

Controversy and complexity – identifying the real ERRβ and its splice variants

In 1999, Chen et al. published a study that called into question the true species of origin for hERR2, which was by now widely accepted as human ERRβ [Chen et al., 1999]. Using an informatics-centric approach, the authors relied on rapidly expanding expressed sequence tag (EST) databases to identify novel nuclear receptor-like sequences, then adapted inverse PCR-based cloning to identify two ERRs, hERRβ2 (GenBank ID AF094517.1, see Table 1) and hERRγ2 from human testis and fetal brain cDNA libraries, respectively. Their hERRβ2 sequence has several key differences with the original hERR2, despite being cloned from the same tissue and having 90% nucleotide and 95% protein sequence identity between most of their open reading frames. hERRβ2 codes for an additional 67 amino acids at the carboxyl terminus, there is no homology between its 5' and 3' untranslated regions (UTRs) and those of hERR2, and Northern blot analysis with probes designed from

Table 1. GenBank Identifiers for ESRRB.

Species	GenBank Identifier #	Transcripts
Human	AF094517.1	hERRβ2
	NM_004452.3	ERRβ
	AY451389.1	ERRβ
	AY451390.1	ERRβ
Mouse	S82458.1	mERR-2
Rat	X51417.1*	hERR2*
	AY383731.1	rERRβ

its 3' UTR identified low but detectable expression of multiple transcripts ranging from 1.0 to 5.5 kb in length in a range of human tissues, including heart, kidney, and liver. The lack of homology between UTRs, coupled with the inability of primers designed against hERR2 to amplify any product from human genomic DNA, demonstrated that hERRβ2 and hERR2 are distinct genes and suggested that the latter might not, in fact, be of human origin. Chen et al. went on to show that hERR2 is actually the rat ERRβ gene, the full sequence of which (GenBank AY383731.1) would not be deposited for another four years, while hERRβ2 is the true human ERRβ [Chen et al., 1999].

Primate-specific alternative splicing

Pre-messenger RNA (pre-mRNA) splicing is a process that is carried out by the spliceosome, a massive multi-protein complex that removes introns and joins together exons into a mature mRNA transcript [Yan et al., 2015]. Alternative splicing (AS) allows a single gene to combine its exons into multiple configurations, and has become the accepted theory as to how eukaryotic cells are able to translate 90,000 proteins from only 25,000 genes [Roy et al., 2013]. The multiple hERRβ2 transcripts identified in [Chen et al., 1999] imply the existence of AS and/or multiple transcriptional start/stop sites for human ERRβ. Consistent with this, their PCR-based validation studies with primers designed towards the amino terminus of hERRβ2 could only detect expression in testis (the tissue from which it was cloned), while hERRβ2 carboxyl terminal primers amplified product in human heart, kidney, and liver, where Northern blot studies show expression.

The existence of ERRβ AS was formally demonstrated in 2006, when Zhou et al. showed that there are at least three distinct human ERRβ variants: the previously identified hERRβ2 and two new forms, ERRβΔ10 (Δ10) and 'short form' ERRβ (ERRβsf, Figure 1A) [Zhou et al., 2006]. At the mRNA level, ERRβ2 includes 12 exons, Δ10 is comprised of exons 3-9; 11 and part of 12, and ERRβsf contains only exons 3-9, terminating at an intronic stop codon within intron 9. At the protein level (Figure 1B), as reported previously, the ERRβ2 protein contains 500 amino acids. The Δ10 protein contains 508 amino acids; a single base frame shift that occurs upon exon 10 exclusion leads to a carboxy-terminal extension of the LBD with less than 5% homology and notable secondary structure differences between it and that of β2 (Figure 1C). The ERRβsf protein is 433 amino acids in length, which is identical to the corresponding domains of ERRβ2 and Δ10 and highly homologous to the mouse and rat ERRβ proteins. In other nuclear receptors, carboxyl terminal extensions known as F domains modulate receptor function and expression [Patel and Skafar, 2015; Skafar and Zhao, 2008]. cDNA exogenous expression studies with ERRβ2, ERRβsf, and Δ10 suggest that these have differing degrees of transcriptional activity on ERE-containing promoter-reporter constructs, and that ERRβ2 shows a greater propensity to localize to the cytoplasm when transfected into COS-1 cells [Zhou et al., 2006]. By contrast, ERRβsf and Δ10 are almost exclusively nuclear-localized. All three ERRβ variants contain a nuclear localization sequence (NLS) in their hinge region or D domain (amino acids 173-190), and Zhou et al. speculate that the F domain of ERRβ2, but not Δ10, counteracts the function of the NLS. It is also interesting to note that while isolating ERRβ from a human kidney cDNA library for the creation of tagged LBD constructs used to characterize synthetic ERR ligands, [Coward et al., 2001] specifically state that the F domain was excluded from the final GST-ERRβ to improve recombinant protein expression. Sequence analysis of the reverse primer used to isolate that cDNA suggests that it is ERRβ2.

Through the use of variant-specific primers, Zhou et al. showed that ERRβsf is expressed in a broad range of human fetal and adult tissues, while ERRβ2 and Δ10 are restricted to testis and kidney. However, their analysis of genomic data for other species, including rodents, zebrafish [Bardet et al., 2004], dogs, and chickens, finds no evidence of sequences corresponding to carboxyl terminal exons 10-12 in these lower organisms. These data strongly suggest that 3' AS of ERRβ is a primate-specific event. This assertion has held up over time as other genomes

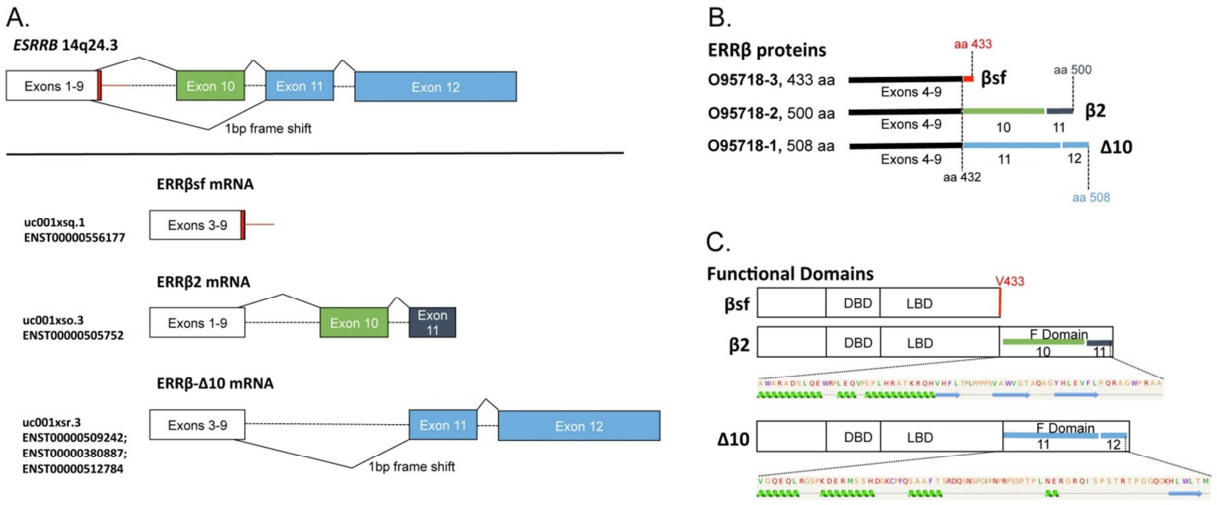

Figure 1. ERRβ splice variants. A, ERRβ mRNA. The ESRRB gene produces three distinct splice variants through inclusion or exclusion of carboxyl terminal exons. UCSC Genome Browser identifiers for each transcript are shown. B, ERRβ proteins. ERRβsf, ERRβ2, and ERRβ-Δ10 variants and corresponding amino acid (aa) differences are shown to scale along with Online Mendelian Inheritance in Man (OMIM) identifiers. C, Functional domains. Common amino acids 1-432 contain well-established DNA- and ligand-binding domains (DBD, LBD). Amino acid sequences and Phyre structural predictions [Kelley and Sternberg, 2009] of the divergent carboxyl terminal F domains of β2 and Δ10 are enlarged.

have been sequenced, e.g. those of killifish and pig [Tarrant et al., 2006; Yang et al., 2015], which show or predict protein products equivalent to ERRβsf.

Annotation errors and the challenge of assessing human ESRRB expression in public gene expression data

The proliferation of large-scale transcriptomic studies, and the publicly-available data they generate, have potential to broaden our knowledge of ERRβ function in contexts where it has not yet been directly studied. However, erroneous use of the 3' UTR from hERR2 (GenBank ID X51417.1) – known to be rat ERRβ [Chen et al., 1999] – for probeset design on human versions of the most popular gene expression profiling platforms is a significant source of confusion. Table 2 shows nucleotide Basic Local Alignment Search Tool (BLASTn) coverage of ERRβ transcript identifiers for selected probeset IDs present on widely-reported Affymetrix and Agilent platforms; target sequences are shown in Supplementary Material. In addition to poor query coverage of known ERRβ sequences, four of these probesets (207726_at and 160036_at for Affymetrix, A_23_P22183 and A_23_P22190 for Agilent) yield zero ESRRB-relevant hits when searched against the Human Genomic + Transcript Collection. This is in direct contrast to probesets 223858_at (Affymetrix), A_23_P391857 (Agilent) and others (not shown), which identify multiple ESRRB-relevant hits and have better query coverage. Probeset 207726_at alone is responsible for >37% of human ESRRB gene expression data in the Gene Expression Omnibus (GEO, 979 records out of 2597). This presents a big problem for data repurposing. End-users may unwittingly draw spurious conclusions from data derived from the wrong probeset, or find no correlation with phenotype or disease state where one might actually exist. This is particularly true for ERRβsf, which with respect to cDNA exogenous expression studies is arguably the best characterized, but only probesets 787_at and 54212_at (Affymetrix U95 series) or A_23_P65597 (Agilent Human 1A Oligo Microarray v2) have any coverage of this splice variant and these platforms are no longer actively produced.

The less biased nature of RNA sequencing (RNAseq) should help to alleviate these issues in the future, but only as this data type is more widely reported and raw data deposited. Table 3 shows transcript identifiers from two sources, Ensembl and the UCSC Genome Browser, for Δ10, ERRβ2, and ERRβsf. Ensembl (release 82) shows eight possible ESRRB transcripts, although three of these mRNAs are not predicted to make a protein. The transcript IDs that show a protein of 433 and 500 amino acids correspond to βsf and β2, respectively, even though the 500 aa ID is annotated as undergoing nonsense-mediated decay. The remaining transcript IDs all show a predicted protein of 508 amino acids, which corresponds to the length of Δ10. While the first two IDs have different annotations of their exons and overall transcript length, their predicted protein contains the same 508 amino acids as the transcribed protein sequence. The last transcript ID also predicts 508 amino acids, but an alternative start site shifts the coding region upstream to include 5 additional amino acids at the beginning of the transcript.

Table 2. Human ESRRB probeset errors on popular gene expression profiling platforms

Source	Probeset ID	Platform(s)	BLASTn Query Coverage
Affymetrix	207726_at	U133A, U133 Plus 2.0	hERR2, X51417.1, 100% hERRβ2, AF094517.1, 11% ERRβ, NM_004452.3, 14% ERRβsf, AY451389.1, 2% ERRβ- Δ 10, AY451390.1, 2% *Zero hits from Human Genomic+Transcript
	160036_at	U95, U95A-Av2, U95B-E	hERR2, X51417.1, 100% hERRβ2, AF094517.1, 9% ERRβ, NM_004452.3, 12% *Zero hits from Human Genomic+Transcript
	223858_at	U133 Plus 2.0	hERR2, X51417.1, 5% hERRβ2, AF094517.1, 100% ERRβ, NM_004452.3, 76% ERRβ-Δ10, AY451390.1, 45% ^Multiple hits from Human Genomic+Transcript
Agilent	A_23_P22183	Human 1A Oligo Microarray v2	hERR2, X51417.1, 100% hERRβ2, AF094517.1, 18% ERRβ, NM_004452.3, 28% ERRβsf, AY451389.1, 18% ERRβ-Δ10, AY451390.1, 18% *Zero hits from Human Genomic+Transcript
	A_23_P22190	Human Genome 44K	hERR2, X51417.1, 100% *Zero hits from Human Genomic+Transcript
	A_23_P391857	Human 1A Oligo Microarray v2, Human Genome 44K	hERRβ2, AF094517.1, 100% ERRβ, NM_004452.3, 100% ERRβ-Δ10, AY451390.1,100% ^Multiple hits from Human Genomic+Transcript

Tool building – ERRβ synthetic ligands

X-ray crystallographic structures of the LBDs of ONRs, coupled with the discovery and synthesis of small molecules that fit within their ligand-binding pocket(s), have accelerated our understanding of the function of these proteins (recently reviewed in [Gallastegui et al., 2015]). However, the direct study of ERRβ in these contexts has been minimal. The LBD of the related ERRγ, crystallized first by Greschik and colleagues [Greschik et al., 2004; Greschik et al., 2002] and refined by several other groups [Abad et al., 2008; Chao et al., 2006; Wang et al., 2006], is at the amino acid level ~80% identical to that of ERRβ (reviewed in [Ariazi and Jordan, 2006]). More recent studies [Collin et al., 2008; Di Micco et al., 2014] have used these ERRγ crystal structures to build homology models of the ERRβ LBD, which to our knowledge has not yet been crystallized. Sequence alignment shows that within the ligand-binding pocket itself, only two of 19 residues differ between ERRβ and ERRγ: in Helix 7, Asparagine 346 of ERRγ corresponds to Tyrosine 321 of ERRβ; and in Helix 5, Valine 313 of ERRγ corresponds to Isoleucine 288 of ERRβ. Given the ~90% identity of amino acid residues within the ligand-binding pockets of ERRγ vs. ERRβ, it is not surprising that these receptors share several synthetic antagonists and agonists (summarized in Table 4).

Table 3. Human ESRRB splice variant identifiers from UCSC Genome Browser and Ensembl.

Database	ERRβsf	ERRβ2β2	ERRβ-Δ10β-Δ10
UCSC Genome Browser	uc001xsq.1	uc001xso.0	uc001xsr.3
Ensembl Relase 82	ENST00000556177	ENST00000505752	ENST00000509242; ENST00000380887; ENST00000512784

Antagonists

The synthetic estrogen diethylstilbestrol (DES) was the first ERRβ antagonist to be identified [Tremblay et al., 2001b]. For thirty years DES was used in pregnant women to prevent miscarriage, with the unintended side effects of increasing the risk of breast and vaginal cancers not only in women who received the drug, but also their female offspring [Harris and Waring, 2012; Hilakivi-Clarke, 2014]. Tremblay et al. hypothesized a connection between DES and ERRβ based on the striking similarity between the placental phenotype of ERRβ-null mice [Luo et al., 1997] (but not ERRα-null or double ERα/ERβ-null animals) and pregnant wild type mice exposed to DES [Scott and Adejokun, 1980] – in both cases, trophoblast differentiation is accelerated, leading to the accumulation of trophoblast giant cells (see below). Using a coactivator displacement assay, in which the biotinylated receptor interacting domain (RID) of steroid receptor coactivator-2 (SRC2, GRIP1) is bound to GST-tagged receptor LBDs and compounds are screened for their ability to enhance or reduce this interaction, they showed that DES is a micromolar (IC50 = 1 μM) antagonist for all three ERR family members. DES antagonist activity was confirmed in cell-based luciferase promoter-reporter assays. Interestingly, this study also identified resveratrol as an ERR antagonist, though a weaker one by at least an order of magnitude [Tremblay et al., 2001b]. The resolution of the crystal structure of the ERRγ LBD bound to DES by Greschik et al. established that the mechanism of antagonism involves displacement of Phenylalanine 435 (common to ERRβ) in the ligand binding pocket, which causes Helix 12 to be dislodged from the LBD [Greschik et al., 2004; Greschik et al., 2002].

4-hydroxytamoxifen (4HT) was initially identified as another ERRβ antagonist. Coward et al. [Coward et al., 2001] and Tremblay et al. [Tremblay et al., 2001a] again used coactivator displacement assays (GST-tagged receptor LBDs and either biotinylated SRC1.2 or receptor interacting protein 140 (RIP140), respectively) to show that 4HT could disrupt receptor/coactivator interaction. However, both groups observed that 4HT is 3-5 times more potent on the LBD of ERRγ than ERRβ, and Coward et al. report that despite the ability of 4HT to disrupt ERRβ LBD interactions with the SRC1.2 peptide in vitro, it can not do so in a mammalian two-hybrid assay in intact cells [Coward et al., 2001]; these data suggest

Table 4. Selected ERR synthetic ligands.

Compound	Category	Receptor(s) Targeted	Other Activities
Diethylstilbestrol (DES)	Antagonist	All ERRs	Estrogen Receptor agonist
Resveratrol	Antagonist	All ERRs	Estrogen Receptor mixed agonist/antagonist
4-hydroxytamoxifen (4HT)	Antagonist	ERRγ	Estrogen Receptor antagonist; Asn vs. Tyr in LBD confers ERRγ specificity
4-methylenesterols, *T. swinhoei*	Antagonist	ERRβ	Pregnane-X-Receptor agonists; Farnesoid-X-Receptor antagonists
Bisphenol A (BPA)	Agonist	ERRγ	Estrogen Receptor agonist; Asn vs. Tyr in LBD confers ERRγ specificity
Soy Isoflavones (genistein)	Agonist	All ERRs	Estrogen Receptor agonist
GSK4716	Agonist	ERRβ, ERRγ	Glucocorticoid Receptor trans-activation; Smoothened Inhibitor
DY131	Agonist	ERRβ, ERRγ	Smoothened inhibitor

that 4HT is not a true ERRβ antagonist. The resolution of the crystal structure of the ERRγ LBD bound to 4HT by Greschik et al. shows that, like DES, it alters the conformation of Helix 12, though it does so by steric hindrance owing to the long side chain of 4HT rather than displacement of a conserved phenylalanine [Greschik et al., 2004; Greschik et al., 2002]. The selective antagonist activity of 4HT for ERRγ has now been specifically linked to one of the two amino acids that differ between it and ERRβ within the ligand-binding pocket; mutation of Asparagine 346 in ERRγ to Tyrosine (the corresponding amino acid in ERRβ) reduces 4HT binding affinity by 4-fold [Liu et al., 2014], identical to the difference in potency observed in [Coward et al., 2001; Tremblay et al., 2001a]. This substitution also significantly impairs binding of the endocrine disruptor bisphenol A (BPA), which is an ERRγ (but not ERRβ) agonist [Takayanagi et al., 2006], further implicating Tyrosine 321 as a key structural determinant of synthetic ligand specificity for ERRβ vs. ERRγ.

Di Micco et al. recently combined homology models of the ERRβ LBD with GAL4-luciferase assays to identify a group of 4-methylenesterols, natural products isolated from the marine sponge Theonella swinhoei, as antagonists for all three ERRs [Di Micco et al., 2014]. These compounds also modulate the activity of other ONRs, including pregnane-X-receptor and farnesoid-X-receptor. Additional compounds may ultimately prove to be ERRβ antagonists, as well. For example, troglitazone and kaempferol inhibit the transcriptional activity of ERRα and ERRγ, and in vitro coactivator binding to the isolated LBDs of these receptors, but ERRβ was not specifically tested [Wang et al., 2009; Wang et al., 2010b].

Agonists

The soy isoflavones genistein, daidzein, and biochanin a increase coactivator recruitment to the LBD of all three ERRs, similar to their effects on classical estrogen receptors [Suetsugi et al., 2003]. Two highly similar synthetic small molecules are known to function more specifically as agonists of ERRβ: GSK4716 and DY131 (also known as GSK9089) [Yu and Forman, 2005; Zuercher et al., 2005]. These acyl hydrazones differ by a single functional group - a terminal isopropyl group in GSK4716 and a terminal diethylamino group in DY131. GAL4-luciferase and ERE-luciferase assays using isolated LBDs or full-length receptors, respectively, show that both compounds increase the transcriptional activity of ERRβ and ERRγ, with no effect on either ERRα or classical estrogen receptors; Zuercher et al. also show that both molecules can compete with radiolabeled 4HT for binding to ERRγ [Zuercher et al., 2005]. Subsequent resolution of the crystal structure of ERRγ bound to GSK4716 and the RIP140 coactivator peptide identified an unexpected molecular mechanism of activation [Wang et al., 2006]. Like many ONRs, the ERRs are constitutively active, with Helix 12 pre-positioned in an active conformation that, together with Helices 3 and 5, forms a groove permissive for coactivator binding

([Darimont et al., 1998; Heery et al., 1997], reviewed in [Gallastegui et al., 2015]). GSK4716 does not alter this conformation directly, but instead shifts the position of amino acids in Helices 1 and 3, enlarging the ligand-binding pocket significantly while also increasing the stability of the LBD [Wang et al., 2006]; these amino acids are conserved in ERRβ, suggesting that the mechanism of activation of this receptor by these ligands is likely similar.

Much as ERRβ antagonists have receptor-independent activities, so too do the agonists DY131 and GSK4716. Wang et al. [Wang et al., 2010a] reported that GSK4716 induces expression of GR and several of its target genes in differentiated mouse skeletal muscle cells, and enhances GR-dependent transcriptional activation of a GRE-containing luciferase reporter. This seemingly contradicts the finding by Trapp and Holsboer that exogenous hERR2 (now known to be rat ERRβ, analogous to human ERRβsf) inhibits GR transcriptional activity [Trapp and Holsboer, 1996]. However, ERRγ-directed RNAi inhibits expression of GR and a subset of target genes in the skeletal muscle cell model, suggesting that ERRγ is the more relevant target for GSK4716 in this setting. More recently, both agonists have been shown to function as inhibitors of Hedgehog signal transduction through direct binding to Smoothened (Smo), which prevents its Sonic hedgehog (Shh)-induced redistribution to the primary cilium and blocks downstream GLI transcriptional activation [Wang et al., 2012]. Ligand competition assays suggest that DY131 antagonizes wild type Smo through the same or a similar mechanism as more established inhibitors like cyclopamine and GDC-0449 (vismodegib), but is unable to suppress the oncogenic SmoM2 mutant. This initially raised the question as to whether our observation of DY131-mediated mitotic arrest in the T98G cellular model of glioblastoma (GBM; see below) is the result of Smo inhibition, but neither cyclopamine nor GDC-0449 cause this phenotype. That, coupled with reversal (and rescue) of the mitotic arrest phenotype by shRNA-mediated depletion of the ERRβ2 splice variant (and re-expression of an shRNA-resistant cDNA in silenced cells) argues against this ‘off target’ effect [Heckler and Riggins, 2015].

Functions of ERRβ in development

ERRβ plays a critical role in the development and normal physiologic function of several tissues and organ systems in the mouse, and a specific form of nonsyndromic hearing impairment in humans. Given that most of these studies have been carried out in mice, it is important to recall that there is only one murine ERRβ, which is homologous to the human ERRβsf splice variant.

Placental development

[Pettersson et al., 1996] first reported the expression of ERRβ in mouse conceptuses at 6.5 days post coitum (dpc) in the ectodermally derived subregion of the amniotic fold. By day 7.5 they detected ERRβ in chorion with highest signal at the boundary between chorion and the extraembryonic ectoderm, suggesting a role in chorion formation in the placenta. At day 8.5 no expression of ERRβ is detected in the basal part of the chorionic plate or any other part of the embryo. This expression pattern of ERRβ disclosed a very specific spatiotemporal role of this receptor in the development of the chorion and the placenta.

Subsequent studies by [Luo et al., 1997] also suggest a very specific expression of ERRβ in extra embryonic tissues during development in mice. They detect ERRβ in the ectodermally derived region of amniotic fluid at 6.5 dpc with no expression of ERRβ by day 9.5, again suggesting that ERRβ plays an important role in the formation of the chorion. ERRβ-/- mice have severe placental abnormalities and die at 10.5 dpc, asserting that ERRβ knockout is embryonically lethal and important for placental formation. These mice show abnormal chorion development and deficiency of diploid trophoblast, which could be rescued by tetraploid wild type embryos, suggesting extra embryonic expression of ERRβ. ERRβ (i.e., ERRβsf) therefore seems to be important in mediating critical function of chorion and terminal differentiation of the diploid trophoblast during placental development in mice. [Tremblay et al., 2001b] show that mice treated with DES from days 4.5-8.5 dpc have a similar phenotype to ERRβ null mice, with placental abnormalities in trophoblast differentiation. DES acts as an antagonist of ERRβ and the authors suggest that DES effects could be through the ERRβ receptor. More recently, [Nagao et al., 2013] treated mice with DES and found that ERRβ expression is observed and does not change with DES treatment. These mice did have placental abnormalities and an increase in trophoblast giant cells, as observed previously by [Tremblay et al., 2001b].

ERRβ also seems to be important in differentiation of primordial germ cells (PGC) in mice. ERRβ is expressed in embryonic tissue at day 13.5, and was detected in both male and female gonads and the brain [Mitsunaga et al., 2004]. However, they did not detect any ERRβ in the placenta or any other tissues at day 13.5. This was the first report of ERRβ expressed in embryonic tissues and not just extra embryonic tissues as reported by [Pettersson et al., 1996] and [Luo et al., 1997]. ERRβ expression in PGCs is diminished by day 15.5, again suggesting a very transient expression and role of ERRβ in germ cell differentiation. ERRβ-/- mice, when rescued by triploid wild type embryos, were normal and fertile but the proliferation of PGCs was affected as determined

by staining for Ki67 and Mitotic Protein Monoclonal #2 (MPM2), which detects mitotic phosphorylation of multiple proteins. ERRβ therefore plays an important role PGCs proliferation during development. Interestingly, these mice have behavioral defects (particularly the females), implying that ERRβ could play a role in the brain.

Recently, [Kumar and Mendelson, 2011] reported that the family member ERRγ is induced during syncytiotrophoblast differentiation and requires oxygen. ERRγ in turn induces the expression of the hCYP19I.1 gene by binding to its promoter. They did not, however, find any expression of any of the isoform of ERRβ in human trophoblast or differentiated placental cells. ERRβ also did not induce the expression of hCYP19I.1 in transient transfection experiments, suggesting a more specific role for ERRγ in human placental development, although the isoform of ERRβ used in these experiments is not clear. This also agrees with [Xie et al., 2009] that ERRβ is undetectable in human embryonic stem cells (see below). ERRβ may therefore have a species-specific role in embryonic differentiation and placental development, as it is abundantly expressed in mouse embryonic cells but not in human embryonic cells. Also, the limited duration of ERRβ expression in both the placenta and the embryonic PGCs suggests that ERRβ effects are restricted to a very brief window during development and attention needs to be given to this while studying the role ERRβ in this setting.

Inner ear development

[Chen and Nathans, 2007] first showed that ERRβ (analogous to human ERRβsf) is expressed in strial marginal cells of the cochlea and the vestibular dark cells of the mouse inner ear. They generated conditional ERRβ-/- mice that exhibit head bobbing and spinning and running in circles, suggesting a defect in vestibular function. These mice have hearing impairment and show characteristics of diminished endolymph production, suggesting the role of ERRβ in development of inner ear. These mice also exhibit a reduction in the expression of ion channels and transporters in inner ear, emphasizing the role of ERRβ in inner ear homeostasis, consistent with the head bobbing and loss of balance phenotype.

In humans, mutations in the ESRRB gene lead to the autosomal recessive nonsyndromic hearing impairment DFNB35. [Collin et al., 2008] have reported recessive nonsyndromic hearing impairment in a large consanguineous family of Turkish origin mapping to chromosome 14q24.3-q34.12 that overlaps with the DFNB35 locus previously reported in a consanguineous family from Pakistan. Linkage and mutation analyses of this region identified alterations in ESRRB exons 5-12, which were also found in the human feta cochlear cDNA library reported by [Luijendijk et al., 2003]. Sequence analysis identified a 7bp duplication in exon 8, which leads to a frameshift and early termination of the protein. Further mutation analysis identified missense mutations in the DNA binding (Alanine110Valine) and ligand binding domains (Leucine320Proline, Leucine347Proline, Proline342Leucine) of ERRβ. More recently, an additional missense mutation (Proline305Histidine) in the ligand-binding domain was identified in another Tunisian family with hearing impairment [Ben Saïd et al., 2011]. The mutants in the ESRRB found in DFNB35 deafness may also contribute to dental decay through the demineralization of the enamel surface, as reported by [Weber et al., 2014]. These mutations are predicted to result in structural changes in DNA binding domain and the ligand binding domain, thereby impairing the overall structural integrity of the protein or affecting the stability of the ERRβ protein leading to functional loss. In human, these missense mutations could affect all three ERRβ splice variants. Two new ERRβ missense mutations - Arginine6Glycine and Arginine382Cystine – have recently been identified by [Wu et al., 2015] in children with good outcomes following cochlear implant installation, and these mutations too should affect all three splice variants.

Collin et al. [2008] analyzed the distribution of ERRβ splice variants at the mRNA level in human tissues by variant-specific PCR, and found that ERRβsf and ERRβ-Δ10 are ubiquitously expressed in all the tissues including the cochlea, while the ERRβ2 is found abundantly only in the testis and the cochlea, with lower expression in retina. The authors suggest that the autosomal recessive hearing impairment might be due to ERRβ2, as it is abundantly expressed in cochlea, although they do not rule out that the other two isoforms could also contribute to the hearing impairment. Overall, ERRβ plays an important role in development of the inner ear and hearing. The mechanism through which ERRβ affects the hearing or function in the inner ear is not known, though it could be through cooperation with TR or GR, as both of these receptors are abundantly expressed in inner ear, and in cell culture studies hERR2 (rat ERRβsf) is known to drive transcription from TREpal [Xie et al., 1999] and suppress GR-mediated transcriptional activity [Trapp and Holsboer, 1996].

Retinal development

[Blackshaw et al., 2001; Blackshaw et al., 2004] reported the expression of ERRβ (functionally, ERRβsf) in photoreceptors of the developing mouse retina by using serial analysis of gene expression (SAGE) and in situ hybridization techniques. They detected expression of ERRβ in rod cells and in immature photoreceptors during the first postnatal

week, but the expression diminishes by postnatal day 10 (P10). Interestingly, during retinal development, ERRβ was expressed at markedly lower levels at prenatal stages, which is very different from its role in embryogenesis, placental development and inner ear development, where it is expressed early in embryo development. The isoform of ERRβ detected is by definition ERRβsf, as this is the only form found in rodents.

Onishi et al. [2010] later reported a more detailed analysis of ERRβ expression in developing and mature mouse retina. They showed that ERRβ is expressed in horizontal cells throughout the first week postnatally and the expression decreases by day P7, similar to what was observed previously. By day P7 they see expression of ERRβ in the outer nuclear layer in the rod photoreceptor cells, but not in cone photoreceptors, as ERRβ colocalized with the rod-specific marker rhodopsin. They also found that ERRβ activated rhodopsin expression and induced expression of rod cell-specific genes such as guanylate cyclase activator proteins and sodium/potassium/calcium rod inner segment cation exchanger Slc24a1 and other genes involved in glycolysis. It is interesting that just like its function in inner ear where ERRβ regulated ion channel homeostasis, it could play a similar role in rod photoreceptor cells by regulating an ion exchanger.

Conditional ERRβ-/- mice have defects in the inner ear but apparently normal retinal development, which agrees with [Blackshaw et al., 2001; Blackshaw et al., 2004], where they see a very low expression of ERRβ during prenatal stages. [Onishi et al., 2010] suggest that ERRβ might play a role in regulation of rod photoreceptor cells at later ages. They found that ERRβ-/- mice show a decrease in the number of rod photoreceptors as they age, and this could be rescued by electroporation of ERRβ. This suggests that ERRβ might have a role in maintaining the rod photoreceptor cells and their function in adults. The authors also suggest that certain individuals lacking ERRβ might suffer from late onset rod photoreceptor degeneration.

Sharon et al., [2002] have reported the expression of ERRβ mRNA in human retinal tissues. However, it is not clear which isoform of ERRβ they detected, though [Collin et al., 2008] have shown expression of the ERRβ2 splice variant to be higher in retinal vs. most other human tissues. Overall, ERRβ seems to play an important role in the maintenance of rod photoreceptors and expression of rod-specific genes in mice, while the role of ERRβ in the human retina remains to be fully elucidated.

Functions of ERRβ in the central nervous system and hypothalamic-pituitary axis

In their genome-wide atlas expression study of adult mouse brain, [Lein et al., 2007] report that ERRβ (i.e., ERRβsf) is expressed in the hindbrain. [Real et al., 2008] performed a subsequent, more detailed study of ERRβ in postnatal and adult mouse brain. Using four different antibodies for ERRβ immunostaining, they show that ERRβ is expressed in hypothalamic suprachiasmatic nucleus, ventral and dorsal geniculate nuclei, pretectal nuclei, superior colliculus, and thalamic posterior nucleus. Most of these regions are targets of retinal projections. This study also shows ERRβ expression in mouse retinal ganglion cells and along the course of retinal axons in the retinorecipient nuclei. Here, ERRβ is detected in both the cytoplasm and nucleus by one of four antibodies used, but the other three antibodies detected only nuclear staining and hence cannot visualize axon fibers. The cytoplasmic expression of ERRβ in axon fibers should be studied further. ERRβ expression in the hypothalamic suprachiasmatic nucleus may suggest that ERRβ plays a role in control of circadian rhythm.

The expression of ERRβ along the efferent retinal projections also agrees with the fact that ERRβ plays an important role in maintenance and function of rod photoreceptors, as discussed above in [Onishi et al., 2010]. The expression of ERRβ in horizontal cells of retina, which are targets of retinal ganglion cells that also express ERRβ, suggests a comprehensive role for ERRβ in mouse retinal development, though connections between these need to be explored further.
[Byerly et al., 2013a] recently showed that ERRβ conditional knockout mice have lower body weights, decreased fat mass, increased metabolic activity and increased energy expenditure, and these differences were observed even at resting metabolic states, suggesting deletion of ERRβ affects energy homeostasis in addition to the vestibular defects observed by [Chen and Nathans, 2007]. These mice also had altered food intake with decreased inter-meal interval, decreased inter-meal satiety ratio and an overall increase in meal number and size. They created a selective knockout of ERRβ (Nestin-Cre: ERRβlox/lox in the hindbrain of the mice, where ERRβ is predominantly expressed in the brain [Lein et al., 2007]. These mice have selectively lower expression of ERRβ in the hindbrain and display similar phenotypes to whole-body ERRβ knockout mice with respect to inter-meal satiety ratio and interval, body weight and metabolic activity. They also have decreased expression of neuropeptide Y (NPY), which is involved in the control of appetite and body weight gain, and had increased insulin sensitivity and lower blood glucose levels. Interestingly, they found that ERRγ played a compensatory role in food intake

and homeostasis when ERRβ is knocked out. The expression of ERRγ is enhanced in ERRβ-/- mice and in Nestin-Cre:ERRβlox/lox mice. When the wild type and ERRβ-/- mice were treated with DY131, a selective agonist of ERRβ and ERRγ, the wt mice displayed similar phenotypes as ERRβ-/- and the ERRβ-/- had further decreases in intra-meal satiety and intra-meal intervals and the expression of NPY. These data suggest that ERRγ can regulate some aspects of food intake in the absence of ERRβ. Together, they both may play an important role in regulating the expression of each other and whole body energy balance and food intake.

In a second paper, [Byerly et al., 2013b] report the effect of ERRβ on response to restrain stress and the hypothalamic-pituitary axis (HPA) with the same animal model system, with the addition of heterozygous-null (Sox2-Cre:ERRβ+/- mice. The heterozygous mice have increased fat mass compared to wt, but homozygous knockout have decreased fat mass and lean mass compared to wt. Surprisingly, the heterozygous mice and homozygous mice do not have any differences in inter-meal satiety ratio, but homozygous mice have lower inter-meal intervals. NPY expression is increased in the heterozygous mice compared to wt, but the highest expression is in the homozygous mice, again suggesting that ERRβ might play an important role in expression of NPY. Considering that heterozygous vs. homozygous deletion of ERRβ have opposing effects on fat mass, this expression pattern of NPY is interesting. The authors speculate that increased fat mass in heterozygous mice is due to increased expression NPY, while in homozygous mice the increased expression of NPY is a secondary effect in response to decreased fat mass.

Acoustic startle tests also show clear differences between heterozygous and homozygous ERRβ knockout animals. Wild type mice had increased cortisone levels in response to stress that returned to baseline after 1 h of recovery. The heterozygous mice have strong response to stress and show elevated cortisone levels compared to wild type animals, and the recovery time was similar between them. The homozygous mice, however, have elevated baseline levels and show no increase in cortisone levels in response to stress; in fact, the cortisone levels decrease in response to stress. The elevated cortisone baseline levels correlated with elevated in situ hybridization staining for Crh (corticotrophin releasing hormone) in the homozygous mice, suggesting ERRβ plays a role in expression of Crh. When the homozygous animals were treated with DY131 there was a small increase in Crh levels in these mice, suggesting that ERRγ can contribute to Crh expression depending on the levels of ERRβ. The expression levels of ERRγ in heterozygous mice is unknown. When the Nestin-Cre:ERRβ mice were subjected to acoustic startle response, these mice showed increased response in this test with a decreased expression of Crh. Therefore, ERRβ might play an important role in the excitatory pathway associated with acoustic startle response. They also showed decrease in corticotrophin releasing hormone 2 (Crhr2), further suggesting that ERRβ plays a role in modulating the hypothalamic axis and the response to stress together with ERRγ.

The glucocorticoid receptor is a potential contributor to the effects of ERRβ (and possibly ERRγ) in the HPA. As discussed above, ERRβ (hERR2, rat ERRβ) inhibits the transcriptional activity of glucocorticoid receptor (GR). The mechanism for transcriptional inhibition of GR by ERRβ is unknown, though ERRβ does not bind to GRE elements or inhibit the binding of GR to DNA, suggesting that it might function as a trans suppressor of GR activity [Trapp and Holsboer, 1996]. On the other hand, ERRγ induces the expression of GR and also promotes the activity of GR [Wang et al., 2010a]. It is interesting that ERRβ and ERRγ, similar in so many ways, have opposing effects on GR activity. Together, ERRβ or ERRγ may contribute to HPA control through their regulation of glucocorticoid receptor and other genes involved in Crh release. In addition, [Ren et al., 2011] show that ERRβ along with ERRα and ERRγ can induce monoamine oxidase, an enzyme that is involved in oxidative de-amination of dopamine. They further find that Parkin, an E3 ubiquitin ligase, binds to all three ERRs and promotes their degradation via the ubiquitin-proteasome pathway. Aberrant ERRβ expression and function may therefore contribute to Parkinson's disease, though to our knowledge this has yet to be specifically studied.

Functions of ERRβ in stem cell biology

Since the cloning and earliest functional studies of murine ERRβ, data have pointed to a role for this receptor in regulating the balance between pluripotency and differentiation (e.g., [Luo et al., 1997; Pettersson et al., 1996; Tremblay et al., 2001b]), and this is one area in which the contributions of ERRβ have been comprehensively summarized by [Papp and Plath, 2012], whose efforts we do not seek to duplicate here. As in the section above, it is important to recall that the mouse has one form of ERRβ, homologous to human ERRβsf.

Seminal papers by [Ivanova et al., 2006], [Loh et al., 2006], and [Zhou et al., 2007a] (GSE4679) identified ERRβ as an essential pluripotency factor in mouse embryonic stem cells (ESCs), a transcriptional target of Oct4 and Nanog whose RNAi-mediated depletion leads to differentiation, and a key regulatory partner for Oct4, Nanog, and Sox2. It is now known that Dax1, another ONR, is an ERRβ target gene in mouse ESCs and establishes a negative feedback

loop through direct repression of ERRβ transcriptional activity [Uranishi et al., 2013]. Chromatin immunoprecipitation and sequencing (ChIP-seq) by [Chen et al., 2008] has identified genome-wide binding sites for ERRβ in mouse ESCs. This ERRβ ChIP-seq dataset (GSE11431) has been subsequently used to develop novel methods for predicting gene expression in this cell type [Ouyang et al., 2009], and as validation for histone modification-directed ChIP-mass spectrometry that show widespread enhancer binding by ERRβ in mouse embryonic stem cells [Engelen et al., 2015]. By contrast, [Xie et al., 2009] demonstrate that while ERRβ mRNA is highly expressed in mouse ESCs, it is undetectable in human ESCs. However, even in the mouse, not all stem cells are created equal, and in trophoblast stem cells (TSCs) ERRβ is a target of FGF signaling, causing its recruitment to a distinct repertoire of target genes and association with distinct coregulatory proteins vs. ESCs [Latos et al., 2015]. Specifically, ERRβ is enriched at the promoters of Eomes and Elf5 in TSCs, and mass spectrometry analysis of ERRβ binding partners in ESCs vs. TSCs shows specific interaction with the histone-modifying (demethylase) enzyme Lsd1 and the Integrator complex in TSCs vs. SWI/SNF and the Mediator complex in ESCs. This suggests that ERRβ makes previously unappreciated contributions to epigenetic regulation through histone demethylation in conjunction with its direct transcriptional activity.

Feng et al. [2009] elegantly showed that ERRβ can substitute for c-Myc and Klf4 in the reversion of mouse fibroblasts to induced pluripotent stem cells (iPSCs) (GSE13190). They further show that ERRγ, but not ERRα, can replace murine ERRβ in iPSC reprogramming. Interestingly, exogenous expression of Gli family zinc finger 1 (GLIS1) can enhance reprogramming in mouse and human iPSCs, but only in mouse iPSCs is this accompanied by an induction of ERRβ [Maekawa et al., 2011]. Coupled with the absence of ERRβ expression in human ESCs [Xie et al., 2009] and the fact that ERRβ appears to be relevant to porcine iPSC reprogramming [Kues et al., 2013], these data suggest that ERRβ is a non-primate regulator of pluripotency. A trio of papers later shed light on the molecular mechanism(s) of ERRβ function in mouse cell pluripotency. ERRβ is a direct target of the Wnt signaling pathway during reprogramming, can substitute for Nanog in this process, and requires a specific coactivator (nuclear receptor coactivator 3 (NCOA3), or amplified in breast cancer 1, AIB1) to exert these effects [Festuccia et al., 2012; Martello et al., 2012; Percharde et al., 2012], and this latter point was supported by a subsequent study [Wu et al., 2012]. Either ERRβ or Nanog can promote pluripotency in ES cells depleted of nucleostemin [Katano et al., 2015] (GSE56797).

A unique feature of stem or pluripotent cells is altered regulation of cell cycle checkpoints, an area in which we now know ERRβ plays an important role (at least in the mouse). In differentiated cells, a robust G1/S checkpoint is required to prevent cell cycle transit in the presence of DNA damage [Ciccia and Elledge, 2010]. However, in mouse ESCs, G1 phase is very short and undergoes differentiation-mediated lengthening. [van der Laan et al., 2013] elegantly showed that expression of the phosphatase Cdc25A remains elevated in mouse ESCs that have experienced DNA damage, tracing this back to maintenance of the deubiquitinating enzyme Dub3 that is a direct transcriptional target of ERRβ. ERRβ knockdown in and DY131 stimulation of mouse ESCs reduces or enhances Dub3 and Cdc25A, respectively, and RNAi-mediated inhibition of Dub3 or Cdc25A led these cells to differentiate. [van der Laan et al., 2014] went on to show that Dub3 expression fluctuates with the cell cycle and is upregulated during S phase in mouse ESCs, but that this is not due to concomitant changes in the expression of ERRβ. Instead, increased expression of the p160 family of nuclear receptor coactivators, and in particular NCOA1, precedes that of Dub3 and is a key regulatory partner for ERRβ in this context. NCOA1 is itself subject to alternative splicing, and the authors demonstrate that both splice variants of this coactivator are able to stimulate ERRβ transcriptional activity and Dub3 expression.

Using RNAseq, [Lu et al., 2015a] have recently analyzed gene expression in NIH3T3 mouse embryo fibroblasts stably expressing exogenous ERRβsf in the absence or presence of Hedgehog ligand-containing conditioned medium (GSE71209). The rationale for this study was to clarify the functional relationship between ESRRB and Hedgehog signaling by identifying ERRβ targets within Hedgehog signal transduction pathways. One hundred nine (109) Hedgehog-regulated mRNAs are modulated by ERRβ overexpression – some genes (Stmn1, Top2a, Hoxd8) are increased in an additive fashion, while for others (Igf1, Smoc2) the relationship between ERRβ and Hedgehog ligand is antagonistic.

Functions of ERRβ in human cancer

A growing number of studies suggest a tumor suppressive role for ERRβ in human cancers, which seemingly contradicts its ability to promote pluripotency and stemness in the mouse. Initially studied in hormone-dependent tumor types, ERRβ splice variants and fusion genes are emerging as contributors to non-epithelial malignancies, though further studies will be required to fully characterize the mechanism(s) by which this occurs.

Prostate cancer

Cheung et al. [2005] first reported that unlike other ERR family members, ERRβ is restricted to normal prostate epithelial cells and some immortalized prostate lines, though it is not clear from the antibody used which ERRβ splice variant(s) is/are being detected. Shortly after alternative splicing of ERRβ became known, [Yu et al., 2008] reported the

expression of ERRβ splice variants in normal immortalized and prostate cancer cell lines by PCR, and performed functional studies using ERRβsf cDNAs. The short form splice variant of ERRβ (ERRβsf) was expressed in all cell lines, while the other two isoforms, ERRβ2 and ERRβ-Δ10, were not expressed in any. Confirming prior studies, expression of the ERRβsf was found to be higher in normal immortalized prostate cell lines than the prostate cancer cell lines. By immunohistochemistry [Yu et al., 2008] further demonstrate that ERRβ is expressed in the nucleus of the epithelial cells and the stromal cells in fetal and pubertal prostate cells, and that expression is decreased with age in adult and aged prostates. ERRβ expression is also downregulated in prostate cancer cells and in premalignant and malignant lesions in clinical prostatic tissues. Ectopic expression of ERRβsf inhibits the growth of LnCAP and DU145 prostate cancer cell lines through the induction of S phase cell cycle arrest, but not apoptosis, and this can be enhanced by the addition of DY131. Further, they report that ERRβsf activates the p21 promoter, as previously shown by [Castet et al., 2006], and that deletion of the first zinc finger of the DNA binding domain blunts this transcriptional activation. [Fujimura et al., 2010] have shown that expression of ERRβ and ERRγ are significantly higher in benign foci vs. cancerous lesions in prostate tissues. However, they find no correlation of ERRβ with the clinical outcome of prostate cancer. Similar to [Cheung et al., 2005], the antibody used to detect ERRβ by immunohistochemistry recognizes an epitope common to all the three splice variants, so it is not clear which variant is being detected in prostate tissues.

Lu et al. [2015b] subsequently used the DU145 prostate cancer model to identify new ERRβ target genes (GSE71208). Cells stably expressing exogenous ERRβsf or the empty vector control were treated with DY131 or vehicle control, and RNAseq was used to identify differentially-expressed targets. Interestingly, DY131 alone had no effect on gene expression in the absence of ERRβ exogenous expression, while ERRβ alone or in combination with DY131 could either upregulate or downregulate putative targets.

Uterine cancer

Using splice variant-specific PCR primers, [Bombail et al., 2008] have shown that ERRβsf and ERRβ2 (referred to as ‘long form’ or ERRβL in this publication) are expressed in normal human endometrium, with ERRβ-Δ10 not detected. ERRβ mRNA levels were specifically quantified at different stages of the menstrual cycle: menstrual, proliferative, early secretory, mid-secretory and late secretory. There were no statistically significant differences between samples observed, though there is a trend towards higher levels in the proliferative and early secretory stages. This study also reported the detection of ERRβ protein in early stage endometrial cancers by immunohistochemistry. Though the antibody used clearly recognizes the ERRβsf exogenously expressed control and exhibits nuclear staining in human tissues, it is not possible to specifically state which endogenous splice variant(s) are being detected.

Bombail et al. [2010] went on to functionally characterize exogenous ERRβsf and ERRβ2 (again referred to as ‘long form,’ or ERRβL) in the ERα-positive Ishikawa endometrial cancer cell line. A key finding of this study is the first demonstration that ERRβ splice variants have differential effects on the transcriptional activity of classical estrogen receptors, in this case ERα. Exogenous ERRβ2 enhances estrogen-induced ERα activity at an ERE-luciferase reporter, while ERRβsf attenuates ERα activity. Using yellow fluorescent protein (YFP)-tagged ERRβ expression constructs and fluorescent recovery after photobleaching (FRAP), they show that ERRβ2 and ERRβsf, have different intranuclear mobility, with ERRβ2 fluorescence recovery being significantly slower. Fluorescence resonance energy transfer (FRET) further implies a physical association between exogenous ERRβ2 and endogenous ERα in response to estrogen. In this study, YFP-ERRβ2 is reported to have predominantly nuclear localization, which the authors point out is different from the initial report by [Zhou et al., 2006] where ERRβ2 is largely cytoplasmic. It is not clear whether cell type-specific factors or the YFP epitope tag contributes to this differential localization.

ERRβsf, which abrogates ERα-driven transcriptional activity in Ishikawa cells [Bombail et al., 2010], also physically interacts with and leads to the downregulation of NF-E2 Related Factor 2 (Nrf2), a key player in the oxidative stress response in this model system [Zhou et al., 2007b]. More recently, [Yamamoto et al., 2012] show that DY131 inhibits the growth of ERα-positive Ishikawa cells, but stimulates that of ERα-negative HEC1A endometrial cancer cells. The authors do not directly address the potential role of ERRβ, though they present evidence that ERRγ exogenous expression phenocopies the effects of DY131 and conclude that ERRγ is therefore the relevant receptor in this context. Further studies are warranted to better define the role of endogenous ERRβ splice variants in endometrial cancer.

Breast cancer`

All three ERRs, when exogenously expressed, are capable of inducing transcription of the estrogen-inducible gene pS2 via the ERE and ERRE in the promoter region [Lu et al., 2001]. This occurs not only in HeLa cells, but also ERα positive and negative

breast cancer cells, with ERRβsf (rat ERRβ) being the most potent inducer. [Ariazi et al., 2002] subsequently utilized quantitative PCR to determine the mRNA levels of the ERRs in human breast tissues and normal mammary epithelium controls; analysis of their ERRβ reverse primer suggests that this primer pair detects all three ERRβ splice variants. ERRβ expression was found to be very low, though in this set of samples it is positively associated with expression of ERβ and inversely associated with the fraction of cells in S phase. Using RNAseq data from The Cancer Genome Atlas (TCGA), [Garattini et al., 2016] show that ESRRB expression (referred to in the manuscript as NR3B2) is significantly reduced in breast tumors vs. normal breast tissue, with lowest expression in the Luminal B and Basal-Like molecular subtypes. These analyses do not appear to distinguish amongst the different ERRβ splice variants.

Sengupta et al. [2014] and [Tanida et al., 2015] recently published two interesting studies with more mechanistic data on the role of exogenously expressed ERRβ splice variants in breast cancer. Sengupta et al. used the YFP-ERRβ2 obtained from [Bombail et al., 2010] (and therefore it is referred to in the manuscript as 'long form,' or ERRβL), while Tanida et al. used a rat ERRβ cDNA (ERRβsf). Both groups find that each isoform of ERRβ can form a complex with ERα, with [Sengupta et al., 2014] further demonstrating that ERRβ2 can interact with ERβ, as well. [Tanida et al., 2015] map the ERα interaction region of ERRβsf to the amino terminus (amino acids 1-92), show that ERRβsf attenuates ERα-mediated transcriptional activity and estrogen-stimulated (but importantly, not basal) MCF7 cell proliferation, and postulate that this is due to restriction of intranuclear mobility of ERα and/or competition for coregulatory proteins, i.e., ERRβ is a direct corepressor of ERα. [Sengupta et al., 2014] performed coimmunoprecipitation experiments with wild type ERRβ2 and ERα in MCF7 cells, in which they find that complex formation is inhibited by estradiol, and with ERRβ2 and ERβ in MDA-MB-231 cells, which is also attenuated by estradiol. In silico 3D molecular modeling approaches suggest that the LBD of ERRβ (selected amino acids from 400 to 429) participates in interactions with ERβ, but that ERRβ/ERα complex formation involves the hinge region or D domain of ERRβ (selected amino acids from 179 to 234). The predictions of these models have not yet been validated by site-directed mutagenesis, which will be required to confirm how these receptors interact in vivo and to resolve differences between these computational models and direct demonstration by Tanida and colleagues that the amino terminus of ERRβ is required for ERα binding. Finally, it is interesting to note that exogenous expression of ERRβsf can apparently suppress the proliferation of a triple negative breast cancer cell line MRK-nu-1 (data not shown, [Tanida et al., 2015]) while having no effect on MCF7 cell growth in the absence of estrogen or on estrogen-independent, Tamoxifen-resistant (yet still ERα-positive) breast cancer cells.

Though its relevance to breast cancer is still emerging, ERRβ has recently been identified as a highly expressed gene in epithelial cells present in human breast milk [Twigger et al., 2015], particularly early in lactation. Interestingly, principal component analysis of differentially-expressed genes identified in this study show that ERRβ variance is opposite to that of KLF4, which it can replace in mouse iPSC reprogramming [Feng et al., 2009]. The contribution of ERRβ to lactation requires further study, particularly given that lactating mammary tissue requires an intricate balance between epithelial cell self-renewal and differentiation.

Glioblastoma

GBM is the most common tumor of the brain, incurable and highly resistant to systemic chemotherapies. Surgery, radiation and adjuvant treatment with the alkylating agent temozolomide are the current standard of care, and result in a median survival of only ~14 months (reviewed in [Prados et al., 2015]). ESRRB is located at 14q24.3, a region frequently deleted in GBMs and lower grade astrocytic tumors [Dichamp et al., 2004; Felsberg et al., 2006; Hu et al., 2002], with Hu et al. showing 47.1% of GBMs in their study to have loss of heterozygosity (LOH) at 14q23-31. Using copy number data from REpository for Molecular BRAin Neoplasia DaTa (REMBRANDT) [Madhavan et al., 2009], now housed within the Georgetown Database of Cancer (GDOC) [Madhavan et al., 2011], we find that patients with ESRRB deletion have significantly worst survival (Figure 2), supporting further mechanistic evaluation of ERRβ in GBM.

We have recently published that ERRβsf and ERRβ2 differentially regulate cell cycle progression in GBM cell lines upon stimulation with DY131, and that this ligand has growth-inhibitory effects in GBM, but not nontransformed, cells [Heckler and Riggins, 2015; Vanacker and Maiorano, 2015]. Growth inhibition is the result of cell cycle arrest and, in cells lacking a functional p53, apoptosis. Intriguingly, the stage at which cell cycle arrest occurs in response to DY131 differs between cell line, and this is the direct result of which splice variant – ERRβsf or ERRβ2 – is dominant. ERRβsf, as shown previously in prostate cancer models by [Yu et al., 2008], drives G1 arrest and the induction of p21, though we also observe senescence, which has not been previously reported. By contrast, ERRβ2 is required for a G2/M arrest that

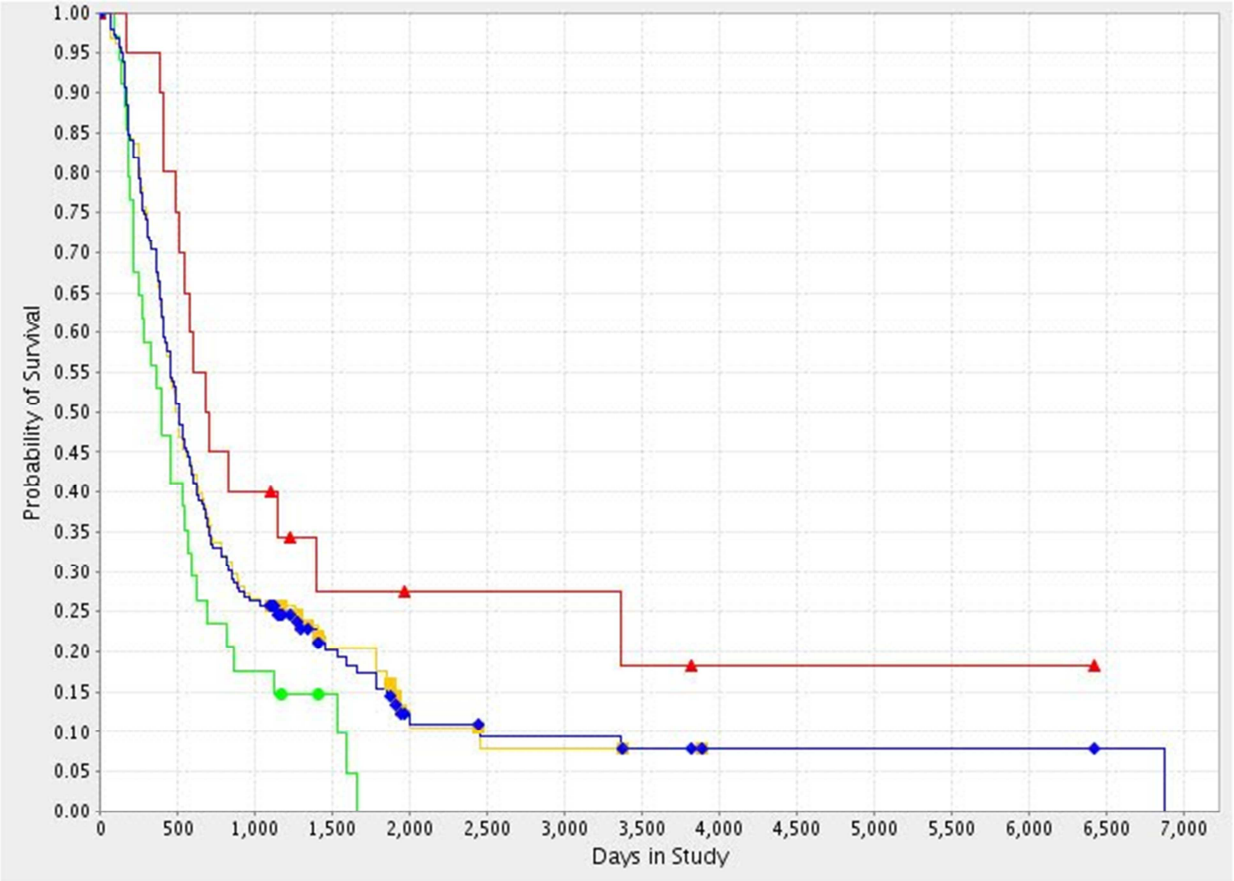

Figure 2. ESRRB copy number and GBM survival. Kaplan-Meier estimation was performed on 'all glioma' Specimens in REMBRANDT using default parameters. Amplified (red) vs. deleted (green) p=0.007; amplified (red) vs . all (yellow) p=0.05; deleted (green) vs. all (yellow) p=0.02.

we hypothesize is due to defective metaphase, given the persistence of Serine 10 phosphorylation on Histone H3. We also demonstrate that ERRβ2 is a dominant-negative inhibitor of ERRβsf-dependent transcriptional activation of the p21 promoter. Our study was the first to identify a function for endogenous ERRβ2 and define its dominant-inhibitory role. Key to this work was our discovery that two distinct monoclonal antibodies (H6707 and H6705, R&D Systems) preferentially recognize endogenous ERRβ2 and ERRβsf, respectively. It is not currently clear why these antibodies should prefer one splice variant to another, though ongoing studies have begun to elucidate the specific epitope(s) recognized. Rational development of ERRβ2 and ERRβ-Δ10 splice variant-specific antibodies based on their divergent F domains should significantly improve the ability our group (and others) to study the expression and function of these splice variants at the protein level.

Pediatric B-cell precursor acute lymphoblastic leukemia (BCP ALL)

Hematologic malignancies are often characterized by genomic rearrangements, with some alteration of the transcription factor paired box 5 (PAX5) affecting ~40% of BCP ALL cases. It was recently reported that an unbalanced t(9;14) rearrangement is a recurrent event in this disease, leading to the production of a PAX5-ESRRB fusion gene [Marincevic-Zuniga et al., 2016; Nordlund et al., 2015]. The protein product of this fusion gene, which joins the amino terminal portion of PAX5 with the carboxy terminal portion of ERRβ, is predicted to retain PAX5 DNA binding and protein/protein interaction activities and contain the full LBD of ERRβ. The breakpoint occurs in intron 4 of ESRRB, which results in the loss of the first zinc finger of the DBD, demonstrated by [Yu et al., 2008] to abrogate receptor DNA binding. It is plausible that PAX5-ESRRB fusions may have alternatively spliced F domains analogous to ERRβ2 and/or ERRβ-Δ10, but the reverse primer used to verify expression of the fusion transcript recognizes a sequence within exon 7, which is conserved in all splice variants. ESRRB expression is absent in ALLs lacking the fusion, implying that the PAX5 promoter is responsible for expression. In addition, ALLs positive for PAX5-ESRRB have a distinct DNA hypomethylation phenotype that is separable from those containing most other PAX5 fusions, and pathway analysis shows enrichment for genes in the Wnt/β-catenin

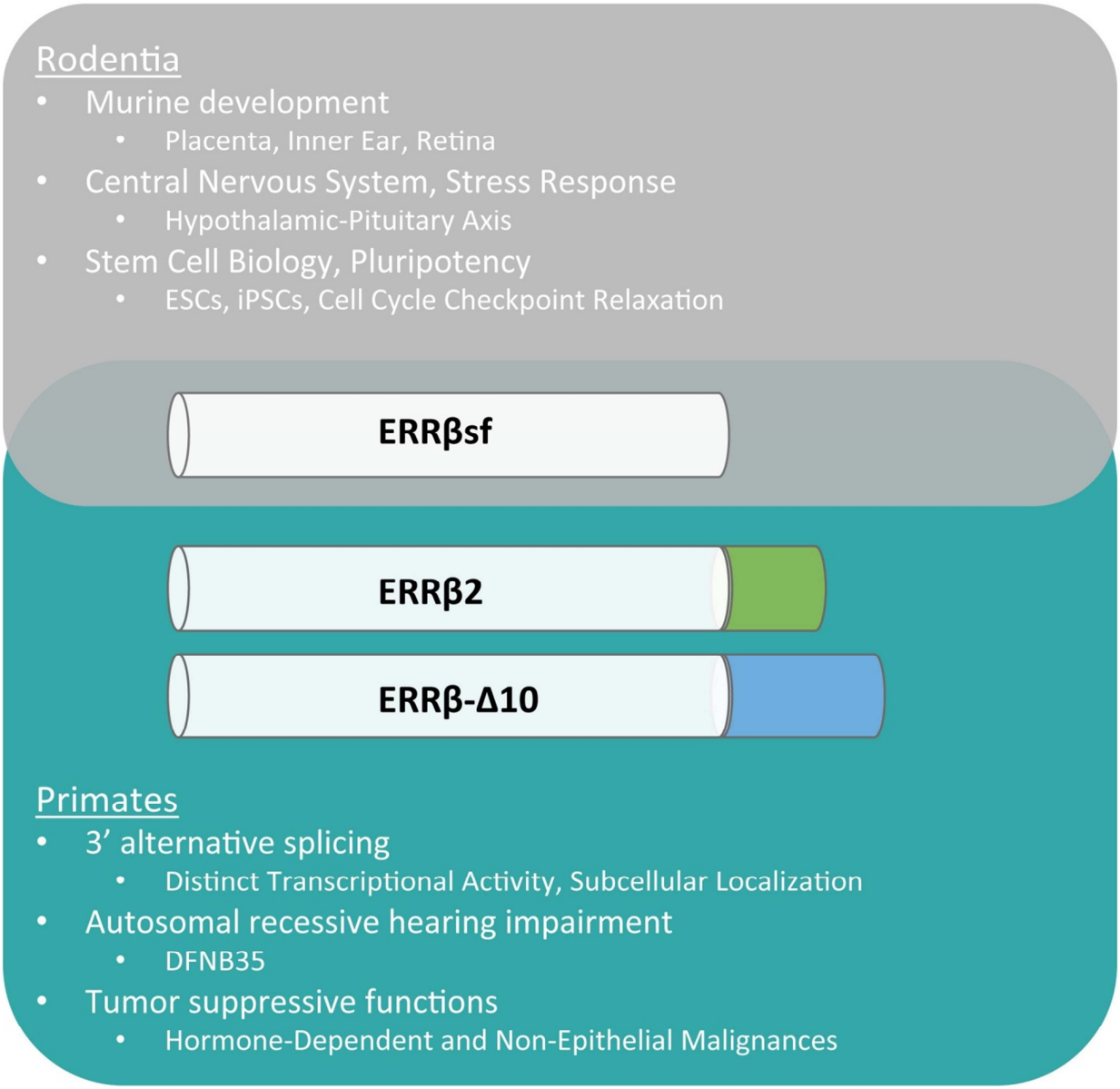

Figure 3. ERRβ and its contributions to diverse biological processes. Key functions of ERRβ splice variants in rodents and primates are summarized.

pathway. This is intriguing, given that in mouse pluripotent cells ERRβ is a target for Wnt pathway-mediated reprogramming [Martello et al., 2012]. Future functional studies will be required to specifically determine the contribution of the ERRβ portion of the PAX5-ESRRB fusion to these events.

Conclusions and future goals

The term 'renaissance' can imply 1) renewed interest or growth in an area previously dormant, and 2) an individual possessing many talents. We think it fair to say that both apply to ERRβ (Figure 3). Key areas of future study and growth in this corner of the ONR field should include: deeper analysis of ERRβ splice variant function and etiology, the latter being completely unexplored; development of ERRβ-selective synthetic ligands that exploit Tyrosine 321 within the ligand-binding pocket; and a clearer understanding of the species-specific functions of this receptor, i.e., how ERRβ behaves as a reprogramming factor in mice vs. a putative tumor suppressor in humans. Alternative splicing is a key source of functional diversity at the proteomic level, and it is likely that we have only just begun to scratch the surface of how this process impinges on the biologic function of ERRβ, and likely other nuclear receptors. It is tempting to speculate that primate-

restricted ERRβ splice variants are responsible for tumor suppressor-like activity in humans, but the fact that we [Heckler and Riggins, 2015] and others [Tanida et al., 2015; Yu et al., 2008] find that ERRβsf is also growth limiting would suggest that the true explanation is not that simple. We have much to learn from the next 27 years of ERRβ-focused research.

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Our goal in writing this review is to provide a comprehensive overview of the collective wisdom of investigators who have been or are currently studying ERRβ in its many forms. We sincerely apologize if, in this effort, we have involuntarily omitted reference to a particular study. We further wish to thank all current and former members of the Riggins lab, and our colleagues within the Lombardi Comprehensive Cancer Center, for their insights and helpful suggestions, particularly Garrett Graham for critical reading of portions of the manuscript. Our work is generously supported by pilot funds from Partners in Research (partnersinresearch.gumc.georgetown.edu) awarded to RBR, developmental funds from the Lombardi Comprehensive Cancer Center (LCCC) Cancer Center Support Grant awarded to RBR (P30 CA051008; PI Dr. Louis M. Weiner), and R21 CA191444 awarded to RBR. Pre-doctoral fellowship funding is provided by the LCCC Tumor Biology Training Grant (DMT; T32 CA009686, PI Dr. Anna T. Riegel) and the Post Baccalaureate Training in Cancer Health Disparities Research Grant from Susan G. Komen for the Cure (AF; PBTDR1222836, PI Dr. Lucile Adams-Campbell). The content of this article is the sole responsibility of the authors and does not represent the official views of the National Cancer Institute, the National Institutes of Health, or Susan G. Komen for the Cure.

Competing interests

The authors have no potential conflicts of interest to disclose.

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Supplementary Material

http://www.nrsignaling.org/nrs14002