Oestrogen Receptor-alpha Regulates Non-Canonical Hedgehog-Signalling in the Mammary Gland

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Abstract

*Mesenchymal dysplasia* (*mes*) mice harbour a truncation in the C-terminal region of the Hedgehog (Hh)-ligand receptor, Patched-1 (Ptch1) and display a block to mammary ductal elongation at puberty. Our lab previously demonstrated that epithelial cell-directed expression of activated c-src rescued this block to mammary development and induced estrogen receptor-alpha (ERα) expression. Using a genetic approach where a conditional allele of ERα was expressed on the *mes* background, we demonstrated that restricted expression of ERα also rescues *mes* mammary morphogenesis with similar kinetics as the MMTV-c-src<sup>Act</sup> mice. We further demonstrated distinct cell-type specific canonical Hh signalling in primary mammary epithelial and mesenchymal cells, and identified a novel Erk1/2 activating Hh signalling system that requires the activities of c-src and ERα, but not Smoothened (smo). These data reveal a novel Hh-signalling cascade operating through c-src and ERα that is required for mammary gland morphogenesis at puberty.
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<th>Description</th>
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<tr>
<td>AF-1</td>
<td>activation function-1</td>
</tr>
<tr>
<td>AF-2</td>
<td>activation function-2</td>
</tr>
<tr>
<td>Alx4</td>
<td>homeobox protein aristaless-like 4</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>Arbp</td>
<td>60S acidic ribosomal protein P0</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
</tr>
<tr>
<td>Boc</td>
<td>brother of Cdo</td>
</tr>
<tr>
<td>BrdU</td>
<td>bromodeoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>Cdo</td>
<td>CAM-related/downregulated by oncogenes</td>
</tr>
<tr>
<td>cFBS</td>
<td>charcoal stripped FBS</td>
</tr>
<tr>
<td>CARM1</td>
<td>co-activator-associated arginine methyltransferase</td>
</tr>
<tr>
<td>CERM</td>
<td>conditional ERα in mammary epithelium</td>
</tr>
<tr>
<td>CHO</td>
<td>chinese hamster ovarian</td>
</tr>
<tr>
<td>CK1α</td>
<td>casein kinase 1α</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA binding domain</td>
</tr>
<tr>
<td>DCIS</td>
<td>ductal carcinoma in situ</td>
</tr>
<tr>
<td>Dhh</td>
<td>desert hedgehog</td>
</tr>
<tr>
<td>disp</td>
<td>dispatched</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>E2</td>
<td>17β-estradiol</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>ERα</td>
<td>estrogen receptor alpha</td>
</tr>
<tr>
<td>ERβ</td>
<td>estrogen receptor beta</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ERKO</td>
<td>estrogen receptor knock out</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>Gas-1</td>
<td>growth arrest-specific 1</td>
</tr>
<tr>
<td>GEN</td>
<td>genistein</td>
</tr>
<tr>
<td>Gli</td>
<td>glioma-associated oncogene</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GR</td>
<td>glucocorticoid receptor</td>
</tr>
<tr>
<td>Grb2</td>
<td>growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>GRE</td>
<td>glucocorticoid response element</td>
</tr>
<tr>
<td>GSK3β</td>
<td>glycogen synthase kinase 3β</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione-s-transferase</td>
</tr>
</tbody>
</table>
HAT  histone acetyltransferase
HCC  hepatocellular carcinoma
HEK  human embryonic kidney
Hh   hedgehog
Hip1 hedgehog interacting protein 1
HRE  hormone response element
Hsp90 heat shock protein 90
IFT  intraflagellar transport
Ihh  indian hedgehog
Kif7  kinesin-like protein
LBD  ligand binding domain
LTR  long-terminal repeat
MAPK Mitogen-activated protein kinases
MEC  mammary epithelial cell
MEK  mitogen activated protein kinase-kinase
mes mesenchymal dysplasia
MMP-9 matrix metalloproteinase 9
MMTV mouse mammary tumour virus
MOER membrane only estrogen receptor
NERKI non-classical ERα knock-in
NLS  nuclear localization signal
N-CoR nuclear receptor co-repressor
NR   nuclear receptor
OHT  4-Hydroxytamoxifen
PBS  phosphate buffered saline
PCR  polymerase chain reaction
PDD  processing determinant domain
PEI  polyethylenimine
Pg   progesterone
PI3K phosphatidylinositol-3-kinase
PKA  protein kinase A
PMSF phenylmethylsulfonyl fluoride
PR   progesterone receptor
PRL  prolactin
Ptch1 Patched-1
RAL  raloxifene
RAR  retinoic acid receptor
REA  repressor of estrogen activity
RNA  ribonucleic acid
RND  resistance, nodulation, and disease
rtTa responsive reverse transactivator
RXR  retinoid x receptor
SANT-1 smoothened antagonist 1
SCC  squamous carcinoma
Scube2 signal sequence, cubulin domain, epidermal growth factor-related 2
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS</td>
<td>sodium-dodecyl-sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SERD</td>
<td>selective estrogen receptor down-regulator</td>
</tr>
<tr>
<td>SERM</td>
<td>selective estrogen receptor modulator</td>
</tr>
<tr>
<td>SH2</td>
<td>src homology 2</td>
</tr>
<tr>
<td>SH3</td>
<td>src homology 3</td>
</tr>
<tr>
<td>Shh</td>
<td>sonic hedgehog</td>
</tr>
<tr>
<td>Smo</td>
<td>smoothened</td>
</tr>
<tr>
<td>SMRT</td>
<td>silencing mediator for retinoid and thyroid hormone receptors</td>
</tr>
<tr>
<td>SP-1</td>
<td>specificity protein 1</td>
</tr>
<tr>
<td>SRC</td>
<td>steroid receptor co-activator</td>
</tr>
<tr>
<td>SSD</td>
<td>sterol sensing domain</td>
</tr>
<tr>
<td>Sufu</td>
<td>suppressor of fused</td>
</tr>
<tr>
<td>TAM</td>
<td>tamoxifen</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>TEB</td>
<td>terminal end bud</td>
</tr>
<tr>
<td>THC</td>
<td>5,11-cis-diethyl-5,6,11,12-tetrahydrochrysene-2,8-diol</td>
</tr>
<tr>
<td>TOT</td>
<td>trans-hydroxytamoxifen</td>
</tr>
<tr>
<td>TR</td>
<td>thyroid hormone receptor</td>
</tr>
<tr>
<td>tetO</td>
<td>tetracycline response operator</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>WW</td>
<td>tryptophan-tryptophan</td>
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</table>
Introduction

1 ESTROGEN RECEPTOR-ALPHA

Estrogens are a class of hormones that play crucial roles in diverse aspects of human biology, such as sexual development and reproduction (Eddy et al., 1996; Lubahn et al., 1993), bone integrity (Chiu et al., 1999; Zittermann et al., 2000), cardiovascular health (Brouchet et al., 2001; Hodgin et al., 2001; Pare et al., 2002) and metabolism (Bryzgalova et al., 2006). The most potent and abundant estrogen in the human body is 17β-estradiol (E2) (Kuiper et al., 1997), and its biological activity is mediated by a group of proteins known as estrogen receptors (ER) (Kuiper and Gustafsson, 1997; Walter et al., 1985).

ERα (Shyamala and Gorski, 1969) and ERβ (Kuiper et al., 1996; Mosselman et al., 1996) are the two ERs that have been identified in humans. These proteins arise from two different genes located on two distinct chromosomes (Enmark et al., 1997; Gosden et al., 1986). Although they show a high degree of overall homology and similarly bind E2 with high affinity (Kuiper et al., 1996, 1997), ERα and ERβ exhibit distinct tissue expression and mediate different physiological functions (Couse et al., 1997; Kuiper et al., 1997). ERα will be the focus of this work.

Classic, or ‘genomic’ actions of E2 are facilitated through ERα acting as a ligand-activated transcription factor (Tora, 1989). Upon E2 binding, ERα modulates transcription by either binding directly to the promoter of E2-regulated genes (Klein-Hitpass et al., 1988; Kumar and Chambon, 1988), or indirectly through protein-protein interactions with other transcription factors (Jakacka et al., 2001; Kushner et al., 2000). More recently, mechanisms by which E2 elicits changes in cellular processes through ERα outside of the nucleus have been identified (for review, see Banjeree et al., 2014)). These ‘non-genomic’ processes are mediated by pools of receptor located at the plasma membrane (Pappas et al., 1995) that cause rapid and transient cellular responses (Bi et al., 2000; Migliaccio et al., 1998, 1996; Stefano et al., 2000).
1.1 ESR1 Gene and Regulation

ERα was the first ER identified (Jensen, 1962; Shyamala and Gorski, 1969). This protein is encoded by the ESR1 gene, which comprises eight exons that span 140 kb (Ponglikitmongkol et al., 1988) on the chromosome 6q25.1 (Gosden et al., 1986; Menasce et al., 1993; Walter et al., 1985). A comparison of the human ERα cDNA sequence with those of chicken (Krust et al., 1986), rat (Koike et al., 1987) and mouse (White et al., 1987) revealed that, with the exception of the 5’ N-terminal region, ERα is highly conserved between species.

Transcription of ESR1 is regulated by multiple promoters in the 5’-flanking regions upstream of the first coding exon 1 (Flouriot et al., 1998; Grandien, 1996; Thompson et al., 1997). Seven transcription start sites (A, B, C, D, E1, F and T1; (Kos et al., 2001)) on six non-translated first exons have been identified for ESR1, as well as at least three ‘non-first’ non-translated exons (E2, T2 and E3) (Grandien, 1996; Ishii et al., 2013; Kos et al., 2000) that do not participate in transcription initiation. All non-translated first exons are capable of splicing to a downstream common acceptor site located on coding exon 1 (Kos et al., 2001). Together, alternative splicing of non-translated exons to this acceptor site results in the production of several variant ERα isoforms that differ only in their unique 5’-untranslated regions (UTRs) (see for review (Hirata et al., 2003; Kos et al., 2001)).

The existence of multiple promoters in ESR1 may facilitate tissue-specific regulation of variant ERα expression through preferential promoter activation (Flouriot et al., 1998; Grandien et al., 1993; Okuda et al., 2003). This was supported by studies showing differential expression of ERα mRNA transcripts in the MCF-7 and ZR-75-1 breast cancer cell lines (Grandien et al., 1993). Differential expression of ERα transcripts were also observed in human breast, uterus, bone and liver tissue (Flouriot et al., 1998; Grandien, 1996; Grandien et al., 1995; Ishii et al., 2013; Okuda et al., 2003), as well as between normal and cancerous breast tissue (Flouriot et al., 1998; Weigel et al., 1995).

The existence of multiple promoters in ESR1 may also be associated with differential ERα requirements during different stages of development. Changes in the levels of variant ERα mRNA
have been observed during embryonic development in the rat brain (Kato et al., 1998). Also, a
study looking at promoter hypermethylation in human endometrial disease reported changes in the
methylation levels of two different ESR1 promoters in uterine cells during distinct phases of the
menstrual cycle (Hori et al., 2000). Taken together, ERα-mediated signalling may be controlled
at the transcriptional level by tight regulation of ESR1 activity. Multiple promoters may allow for
temporal and spatial control of ERα expression by initiating transcription in response to specific
regulatory factors and cellular contexts.

1.2 Synthetic Ligands – SERMs and SERDs

Estrogen receptors are unique among the steroid receptors in their ability to accommodate
binding of a wide variety ligands. This promiscuity can be attributed to the large size of the ligand
binding cavity in the LBD (Brzozowski et al., 1997), as well as its structural plasticity (Nettles et
al., 2007). For this reason, ER activity at the LBD has been pharmacologically exploited, leading
to the development of synthetic ligands ("anti-estrogens") that are used for the treatment of breast
cancer (for review, see (Jordan and Brodie, 2007)). Endocrine therapy aims to inhibit estrogen
signalling in ERα-positive breast cancer cells to stop cell proliferation and/or to induce cell death.
Two types of anti-estrogens with distinct mechanisms of actions have been developed: Selective
Estrogen Receptor Modulators (SERMs) and the Selective Estrogen Receptor Down-regulators
(SERDs). SERMs, including tamoxifen (TAM) (Cole et al., 1971) and raloxifene (RAL) (Fuchs-
Young et al., 1995), exhibit tissue-specific ER agonist or antagonist activity (Vogel et al., 2006;
Wu et al., 2005). On the other hand, SERDs, represented by ICI 164 384 (Wakeling and Bowler,
1987) and ICI 182 780 (fulvestrant) (Wakeling et al., 1991), are pure anti-estrogens that possess
no agonist effects (Howell, 2006).

1.3 ERα Structure and Function

ERα is a member of the large nuclear receptor (NR) superfamily of transcription factors
(for review, see (Gronemeyer et al., 2004)). This mammalian family comprises of more than 45
members, including, but not limited to: receptors for steroid hormones, which includes ER (ERα and ERβ), as well as the androgen receptor (AR) and the glucocorticoid receptor (GR); receptors for non-steroidal ligands, such as the thyroid hormone receptor (TR) and the retinoic acid receptor (RAR); as well as receptors that bind to products of lipid metabolism. Human ERα is encoded by a 595 amino acid polypeptide with a predicted molecular mass of approximately 66 kDa (Green et al., 1986b; Walter et al., 1985).

Members of this nuclear receptor superfamily share a common architecture that harbours distinct structural and functional domains (for review, see (Gronemeyer et al., 2004)). ERα can be subdivided into six regions (termed A-F) (Figure 1) (Green et al., 1986a; Greene et al., 1986). The central DNA-binding domain (DBD) facilitates recognition and binding to estrogen response elements (EREs) located in the promoter regions of target genes (Kumar et al., 1986). The DBD is followed by the D domain that behaves as a flexible hinge between the C and E domains, and contains the nuclear localization signal (NLS). Two activation functions (AFs) facilitate receptor activation (Kumar et al., 1987; Tzukerman et al., 1994). The constitutively active, ligand-independent activation function (AF-1) is contained in the variable 5’ N-terminal A/B domain (Enmark et al., 1997; Tora, 1989), while the ligand-dependent activation function (AF-2) is located within the ligand-binding domain (LBD) in 3’ C-terminal E domain (Tora, 1989). The very C-terminal part of ERα molecule also contains the F domain, which exerts a complex modulatory role on activity of the receptor (Skafar and Zhao, 2008). The structural and functional contributions of the highly conserved DBD and LBD, as well as the AF-1 and AF-2, in ERα transactivation will be discussed in detail below.

Figure 1. The structure of estrogen receptor α (ERα). The 595 amino acid receptor comprises of six nuclear receptor structural domains (A–F) that include activation function 1 (AF1) and AF2 domains, a conserved DNA-binding domain (DBD) and a ligand-binding domain (LBD).
1.3.1 DNA Binding Domain

Nuclear receptors regulate transcription of target genes by directly binding to specific DNA sequences, called hormone response elements (HREs) (Nääär et al., 1991). These sequences are composed of two hexameric half-sites that are separated by spacer sequences of varying lengths. Correct recognition of HREs by their cognate receptor depends on the orientation and sequence of the half site, as well as the spacer length between them. ERs recognize and bind to EREs, which consist of palindromic half sites separated by three intervening base pairs (Klein-Hitpass et al., 1988).

The specificity of the DBD in targeting ERα for gene regulation was demonstrated by mutagenesis experiments in which the DBD of ERα was switched with that of the GR. This chimeric receptor bound to glucocorticoid response elements (GREs) and up-regulated transcription in response to E2 (Green and Chambon, 1987). The DBD of ERα contains two zinc-finger motifs that facilitate DNA binding and receptor dimerization (Kuiper et al., 1997). Mutagenesis experiments revealed that HRE discrimination by their cognate nuclear receptor is dependent on several key amino acids found within these motifs (Green and Chambon, 1987; Mader et al., 1989). Three amino acids (Glu25, Gly26 and Ala29; termed the ‘P Box’) on the first zinc-finger of ERα are responsible for discriminating between differences in half site sequences. Replacement of these amino acids with the corresponding residues of GR changed the specificity of ERα to GRE in GR target genes (Mader et al., 1989). Further investigation identified that five residues (Pro44 to Gln48) on the second zinc-finger motif that are also involved in DNA binding by discriminating half-site spacing lengths in EREs (Martinez and Wahli, 1989; Umesono and Evans, 1989).

ERα acts primarily through binding to EREs as dimers (Beato et al., 1989), with each receptor monomer recognizing one of the two half sites in the response element. Although the major site of receptor dimerization is found in the C-terminal LBD (Fawell et al., 1990), the DBD has also been found to possess weak dimerization properties (Kumar and Chambon, 1988; Tsai et al., 1988). A second region within the DBD, termed the ‘D Box,’ has been implicated in facilitating protein-protein interactions between ERα monomers during DNA binding (Schwabe et al.,
Although isolated DBDs only exist as monomers in solution, assembly of ERα DBDs at EREs is highly cooperative and favours dimeric binding (Schwabe et al., 1993b, 1990; Tsai et al., 1988). Taken together, through the central DBD, ERα employs complex molecular strategies to recognize specific binding sites on target genes to facilitate the correct receptor configuration and DNA-interactions required to drive proper transcription in response to ERα activation.

1.3.2 Ligand Binding Domain

The ligand binding domain (LBD) is found within the E region of ERα and facilitates ligand-docking, dimerization and interactions with co-regulatory factors (Brzozowski et al., 1997). The LBD is arranged into a multi-layered antiparallel “sandwich” comprising of twelve alpha-helices (H1-H12) organized into a hydrophobic core, a ligand-binding pocket, and a transcriptional co-regulator binding surface (Brzozowski et al., 1997; Wurtz et al., 1996). H5/6, H9 and H10 make up the central core of the domain, which is surrounded by two additional layers comprising of helices H1-H4 and H7, H8 and H11. The E2 binding cavity is completely separated from the external environment and takes up a large portion of the hydrophobic core. In addition, H12 and a double stranded β-sheet flank the multi-layered motif near the ligand-binding cavity. H12 is an essential component of AF-2, which regulates ligand-dependent transcriptional activation through the recruitment of co-regulatory factors (Brzozowski et al., 1997; Danielian et al., 1992; Henttu et al., 1997).

1.3.2.1 Recruitment of Co-Activators

The resolution of the crystal structures of several ligand-free (apo) and ligand-occupied (holo) nuclear receptor LBDs have provided molecular details of the various ligand-induced changes in receptor conformation that facilitate receptor-mediated transcriptional activity (Egea et al., 2000). The “mouse trap” mechanism of transitioning from a transcriptionally inactive receptor to an active one was first derived from comparing the E region of apo-RXR to that of holo-RAR
(Parker and White, 1996; Renaud et al., 1995), but has since been described in the activation of ERα (Brzozowski et al., 1997; Eiler et al., 2001; Shiau et al., 1998). Binding of a pure agonist, such as E2, results in a conformational change in the LBD that displaces H12 so that it lies over the ligand-binding pocket. In the E2-ERα complex, without directly interacting with the ligand, H12 sits snugly over the ligand-binding cavity and forms a ‘lid’ (Darimont et al., 1998; Shiau et al., 1998). This ‘active’ positioning of H12 mediates transcriptional activation by sealing the ligand-binding cavity and generating a functional AF-2 that is capable of interacting with co-regulatory factors (Brzozowski et al., 1997).

To gain full transcriptional activity, agonist-bound ERα recruits various transcriptional co-regulators, many of which have various enzymatic activities to modify chromatin structures or to interact with the general transcription apparatus (for review see (Heldring et al., 2007)). The p160 family of proteins (or steroid receptor co-activators (SRCs)), which include three homologous members: SRC-1, SRC-2, and SRC-3, are the best characterized co-activators of ERα (Cheskis et al., 2003; Li and Shang, 2007). These proteins directly bind to a hydrophobic co-activator interacting surface in the ERα LBD formed by the repositioning of H3-H5 and H12 in response to agonist-binding (Darimont et al., 1998; Shiau et al., 1998). These co-activators harbour a conserved hydrophobic helical motif, termed the ‘nuclear receptor (NR) box,’ that contain the LxxLL (where L is leucine and X is any amino acid) core consensus sequence (Heery et al., 1997; Torchia et al., 1997). These sequences have been shown to directly bind to the interaction surface generated by H12 in response to agonist-binding (Darimont et al., 1998; Heery et al., 1997). McInerney et al. reported differential requirements for LxxLL sequences within the NR box of SRC-1 for the activation of ERα, TR and RAR (McInerney et al., 1998). SRC-1 contains three LxxLL sequences within its NR box, and while the interaction of the second LxxLL motif with ERα was sufficient for its activation, TR and RAR activation required interactions with both the second and third LxxLL motifs. Therefore, co-activator binding specificity to different nuclear receptors may be mediated through various interactions of LxxLL sequences within the NR box and the receptor.

In the presence of an agonist, H12 is configured to form a ‘charge clamp’ in which a
conserved glutamate in H12 and a conserved lysine in H3 in the LBD of ERα form hydrogen bonds with the peptide backbone of the LxxLL helix (Heery et al., 1997). These interactions are required for anchoring of the co-activator, as loss or mutation of the glutamate or lysine in the charge clamp prevents transcriptional activation by ERα (Feng et al., 1998).

1.3.2.2 Recruitment of Co-Repressors

Unliganded RAR and TR repress basal transcription in the absence of their cognate ligands (Hörlein et al., 1995; Nagy et al., 1997). This silencing function is mediated by two large nuclear proteins, SMRT (silencing mediator for retinoid and thyroid hormone receptors) and N-CoR (nuclear receptor co-repressor), which when bound, recruit histone deacetylase complexes to the promoter regions of target genes (Alland et al., 1997; Nagy et al., 1997). These co-repressors interact with nuclear receptors through an elongated helix, referred to as the CoRNR box, that contains the conserved consensus sequence, LxxI/HIxxxI/L (Hu and Lazar, 1999). This motif occupies the same hydrophobic interaction surface that is contacted by the LxxLL motif in co-activators upon agonist-binding (Hu and Lazar, 1999; Nagy et al., 1999). In the absence of ligand-binding, the extended helix of the CoRNR box can displace H12 and bind to the hydrophobic groove. However, upon agonist-binding, the co-repressor dissociates from the interaction surface, as its extended helix is too long to be accommodated in the interaction groove when H12 assumes the ‘charge clamp’ configuration (Perissi et al., 1999). Thus, agonist binding reduces the affinity of nuclear receptors for CoRNR box-containing co-repressors and increases the affinity for LxxLL containing co-activators.

Although co-repressors bind to some nuclear receptors in the absence of their cognate ligands, this is not the case for ERα, which is bound to by co-repressors in the presence of an antagonist (Lavinsky et al., 1998; Shang et al., 2000). Both TAM and RAL have been shown to bind to and recruit NCoR and SMRT to a number of ERα target gene promoters (Shang et al., 2000). Lavinsky et al. observed NCoR and SMRT-dependent antagonistic effects of ERα when bound to by the partial antagonist, trans-hydroxytamoxifen (TOT), in MCF-7 cells, that was lost
when cells were treated with anti-NCoR or anti-SMRT IgG. Furthermore, the acquisition of TAM resistance in MCF-7 cells that were grown in athymic nude mice (a model of human breast cancer) was accompanied by a decrease in NCoR levels (Lavinsky et al., 1998).

The repressor of estrogen activity (REA) is an ER-specific co-repressor (Montano et al., 1999). REA was cloned using a dominant-negative form of ERα, which is altered in its C-terminal AF-2 region, as bait in a yeast two-hybrid screen. This group showed, through a GST-pull down assay, a direct interaction of the full-length REA with ERα, and that this interaction was ligand-dependent. REA demonstrated preferential interactions with either the dominant negative ERα or with the antagonist-bound ERα. Furthermore, this co-repressor is specific for ER, as no interactions were observed between PR-B and REA.

1.3.2.3 Differentiation between Agonists and Antagonists

It has been demonstrated that different ligands induce different conformational changes of ERα that influence cofactor binding (Brzozowski et al., 1997; Gangloff et al., 2001). ERα ligands, regardless if they have agonistic or antagonistic effects on ERα, bind to the same ligand-binding cavity in the LBD (Pike et al., 2001). The first solved crystal structures of ligand-bound ERα was of the ERα LBD in complexes with E2 and the selective E2 antagonist, RAL (Brzozowski et al., 1997). In E2-ERα complex, E2 binds diagonally across the cavity between H11, H3, and H6, allowing ERα to adopt a low-energy conformation with H12 sitting over the ligand-binding cavity. In the case of the RAL-ERα complex, the bulky side chain of RAL is unable to fit within the ligand-binding cavity. The side chain protrudes out of the binding pocket and displaces H12, preventing H12 from assuming the ‘agonist’ position. This positioning of H12 inhibits receptor activation by preventing the proper assembly of the co-activator recognition surface. Consistent with genistein (GEN) acting as an ERβ partial agonist (Barkhem et al., 1998), in the GEN-ERβ complex, H12 is bound over the ligand-binding cavity in a position such that it only partially occludes the co-activator recognition surface (Manas et al., 2004; Pike et al., 1999). In contrast, when bound to by the pure antagonist, ICI 164 384 (Barkhem et al., 1998), the bulky side chain
completely occludes the co-activator interaction surface of ERβ, thus completely disordering H12 (Pike et al., 2001).

Not all antagonists possess bulky side chains to sterically hinder the agonist positioning of H12. THC (5,11-cis-diethyl-5,6,11,12-tetrahydrochrysene-2,8-diol) acts as an ERβ antagonist (Sun et al., 1999). Unlike GEN, THC does not possess a bulky side chain, and when bound to ERβ, does not cause the relocation of H12 so that it occupies the co-activator interaction groove (Shiau et al., 2002). THC appears to antagonize ERβ by affecting key residues in the ligand-binding pocket that leads to the stabilization of a transcriptionally non-productive receptor conformation in which H12 does not assume the ‘agonist’ position. This mode of antagonism has been referred to as ‘passive antagonism,’ and may be, to some extent, behind the partial antagonistic proper of certain ER ligands (Greene et al., 2004; Shiau et al., 2002). Mutagenesis studies of the ligand-binding cavity constituents have demonstrated how certain molecular interactions between the ligand and LBD are essential for stabilizing the receptor in the agonist conformation (Gangloff et al., 2001; Gao et al., 2012). If these are disrupted, the receptor structure is shifted toward an alternative nonproductive conformation, and results in changes in transcriptional activation ability. Partial agonists change the conformation of the receptor in a way that is sufficient for co-repressor dissociation, but does not permit the stabilization of AF-2 in the ‘active’ form (Gangloff et al., 2001; Geistlinger et al., 2004; Shiau et al., 2002). These studies have shown that the promiscuous nature of the ERα LBD can support not only the binding of various synthetic and naturally occurring ligands, but also facilitates graded cellular responses elicited by these ligands through tightly regulated associations with co-regulatory factors.

1.3.3 *Activation Functions (AF-1 and AF-2)*

Ligand-induced transcriptional activation of ERα involves the action of two AFs (Lees et al., 1989; Tora, 1989). In contrast to the previously described ligand-dependent AF-2, AF-1 is located within the variable N-terminal A/B domain and operates independently of ligand binding (Lees et al., 1989). The relative contribution exerted by AF-1 and AF-2 on the transcriptional
activity of ERα varies in a promoter- and cell type-specific manner (Tzukerman et al., 1994). Although AF-1 and AF-2 are capable of acting independently, synergy between both are often required for full transcriptional activation of ERα (Kobayashi et al., 2000).

Depending on the cellular context, phosphorylation of ERα can aid in receptor response to ligand stimulation, or can facilitate ligand-independent transactivation (for review, see (Arnal et al., 2013)). Several serine residues have been identified, the majority of them found within the A/B domain, that when phosphorylated play key roles in the activation of AF-1, and therefore, ERα signal transduction (for review see (Le Romancer et al., 2011)). The major phosphorylation sites of ERα include serine 104, 106, 118, and 167 (Figure 2). These residues can be phosphorylated by a number of growth factors, both in the presence and absence of ligand-binding. Phosphorylation site mapping has demonstrated that Ser118 is phosphorylated by Erk1/2, resulting in stimulation of ERα activity (Bunone et al., 1996; Kato et al., 1995). The requirement of this phosphorylation event for transactivation was demonstrated by the reduced ERα-mediated transcription in cells expressing an ERα variant harbouring a point mutation that replaced Ser118 with Ala (S118A) (Joel et al., 1998a).

![Figure 2. Schematic of phosphorylation sites of ERα.](image)

Ligand-independent activation of ERα may also be mediated by phosphorylation of these residues. For example, EGF phosphorylates Ser118 through the Ras-MAPK pathway independent of ligand-binding (Chen et al., 2002). This phosphorylation, and resulting transcriptional activation, has been observed in cells expressing a truncated ERα lacking AF-2. Therefore, in this case, AF-1 activation is sufficient to drive transcription in response to EGF (Bunone et al., 1996). Similar
ligand-independent ERα activation was demonstrated in ovariectomized mice, where uterine proliferation, which is normally E2-dependent, was mediated by EGF (Ignar-Trowbridge et al., 1992).

Several studies have confirmed tissue-specific roles for AF-1 and AF-2 in ERα activity in vivo. Mice deficient in AF-1 (ERαAF-1°) revealed that while dispensable for various vasculoprotective actions of E2, such as NO production (Billon-Gales et al., 2011), AF-1 plays a crucial role in uterine cell proliferation (Abot et al., 2013). It has been suggested that independent activity of AF-1 and AF-2 may be responsible for the tissue-specific effects of SERMs (Arao et al., 2011). It has been shown that TAM inhibits AF-2 activity without inhibiting AF-1, and therefore, may act as a partial agonist in cell types where AF-1 is the dominant activation function regulating receptor activity (Berry et al., 1990). Using a mouse model harbouring two point mutations introduced into H12 that abrogates AF-2 activity (termed the AF2ERKI mouse), Arao et al. demonstrated that TAM induces endometrial proliferation and ERα-mediated gene responses in the absence of AF-2 function (Arao et al., 2011). In contrast, proliferation induced by both E2 and the partial agonist, OHT (4-hydroxytamoxifen) was lost in ERαAF-1° mice. Thomas et al. further demonstrated that phosphorylation of specific serine residues on AF-1 by the MAPK pathway contributes to OHT-mediated ERα activation (Thomas et al., 2008). A reporter gene assay showed that alanine substitution of Ser 104, 106 or 118, or treatment with the MEK inhibitor, U0126 inhibited the OHT agonistic effects on ERα.

2 ESTROGEN RECEPTOR-ALPHA SIGNAL TRANSDUCTION

2.1 Genomic Action of ERα

Classic, or ‘genomic’ actions of E2 are mediated by nuclear localized ERα (for review, see (Chakrabarti et al., 2013)). Upon ligand binding, ERα dimerizes and binds with high affinity to EREs of target gene promoters and induces transcriptional activation. Transcriptional activation is accomplished by the assembly of basal transcriptional complexes that contain RNA polymerase II and other general transcription factors. In the absence of ligand-binding, ERα is maintained
in a stable, ligand-binding competent conformation through its association with Hsp90 (Heat shock protein90)-based chaperone complexes in the nucleus (Lee et al., 2002). This association appears to regulate ERα stability, as Hsp90 disruption induces rapid ERα degradation through the ubiquitin-proteasome pathway (Bagatell et al., 2001; Lee et al., 2002). Ligand binding causes ERα to dissociate from Hsp90 and form DNA-binding homodimers or heterodimers, depending on the cellular context and the bound ligand (Cowley et al., 1997).

The SRC-1/p160 family of co-activators were the first identified and are the best characterized family of co-activators that interact with ERα. Members of this family, SRC-1 (Oñate et al., 1995), SRC-2 (Hong et al., 1996; Voegel et al., 1996) and SRC-3 (Li et al., 1997; Takeshita et al., 1997) facilitate transcriptional activation through direct and/or indirect recruitment of other co-activators that show chromatin-remodeling and histone modification functions (for review, see (York and O’Malley, 2010)). In addition, SRC proteins also possess intrinsic, but weak histone acetyltransferase (HAT) activity (Chen et al., 1997; Spencer et al., 1997). Although all three SRCs directly interact with ERα, they recruit different complexes of secondary cofactors to enhance the transcriptional regulatory activity of the ERα apparatus (Torchia et al., 1997). Such recruited proteins include the CBP (CREB binding protein)p/300 family of co-activators and acetyltransferases (Torchia et al., 1997; Voegel et al., 1998), as well as the protein modifying enzyme, CARM1 (co-activator-associated arginine methyltransferase) (Jimenez-Lara et al., 2000).

A direct interaction between SRC-1 and both AF-1 and AF-2 of ERα has been demonstrated (Webb et al., 1998). The association with AF-1 occurs through a C-terminal interaction domain of SRC-1 that is distinct from the LxxLL recognition motif that interacts with AF-2 (McInerney et al., 1998). In addition to SRC-1, the AF-2 associated co-activator, CBP/p300, was also found to directly bind to AF-1 and potentiate transcriptional activity (Hanstein et al., 1996; Kobayashi et al., 2000). These dual interactions of AF-1 and AF-2 with separate domains of co-activators provides a mechanistic explanation for the observed synergism between both AFs in ERα transactivation.

Not all E2-regulated genes contain EREs (Carroll et al., 2006). In these cases, ERα requires a second DNA-binding transcription factor to mediate its association with promoter sequences
and drive transcription (for review, see Heldring et al., 2011)). E2-dependent regulation of genes harbouring AP-1 (activator protein 1) (Paech et al., 1997; Umayahara et al., 1994) and SP-1 (specificity protein 1) (Saville et al., 2000) binding sequences have been described. E2-bound ERα can be brought these target genes by ‘tethering’ to other transcription factors that bind to these sites (Paech et al., 1997; Umayahara et al., 1994). SP-1 and AP-1, which bind to the Sp1 and AP-1 sites, respectively, are the primary transcription factors that mediate the association of ERα and ERE-free DNA (O’Lone et al., 2004). Binding of Sp1 and Jun/Fos to their respective binding sequences are required for ERα action, and their transcriptional activity, as well as co-activator recruitment, is enhanced by ERα binding (Sun et al., 1998). An intermediary mechanism has also been described whereby estrogen receptors bind to one or two ERE-half sites, and this interaction is stabilized by transcription factor binding at a downstream AP-1 (Wang et al., 2011) or SP-1 site (Petz and Nardulli, 2000).

To assess the importance of classical ERα signalling, a non-classical ERα knock-in (NERKI) mice model was generated and has been used to characterize the in vivo roles of the ERE-independent pathway in several tissues, including bone (Jakacka et al., 2002) and in the female hypothalamic-pituitary-gonadal axis (Glidewell-Kenney et al., 2007). These mice express an ERα mutant that harbours two amino acid substitutions (E207A/G208A) in the first zinc finger of the DBD and fail to bind EREs. In vitro, this mutant receptor failed to activate reporter constructs containing EREs, but were able to regulate transcription from an AP-1 site (Jakacka et al., 2001). Results from these studies suggest that E2 action is mediated by complex crosstalk between ERE-dependent and independent ERα signalling pathways.

2.2 Non-Genomic Actions of ERα

In addition to nuclear, ‘genomic’ activity, ERα is capable of mediating E2 signal transduction outside of the nucleus (for review, see Heldring et al., 2007). These ‘non-genomic’ effects of E2 are facilitated by the activation of an intracellular pool of ERα located at cell surface (Adlanmerini et al., 2014; Pappas et al., 1995; Pedram et al., 2009). Membrane-associated ERα can mediate rapid
cellular responses that are not observed with nuclear ERα activation (for review, see (Banerjee et al., 2014)). Instead of mediating cellular processes through changes in E2-target gene transcription, this form of ERα activity is associated with the activation of various protein kinases. Furthermore, while transcriptional regulation can take hours following E2 exposure, these rapid cellular effects can occur within seconds to minutes.

Several E2 actions mediated by non-nuclear ERα have been described (Kousteni et al., 2001; Zhang et al., 2002). For example, Kousteni et al. demonstrated that E2 stimulation of HeLa cells expressing only a membrane-targeted ERα E domain induces Erk1/2 phosphorylation and supports cell survival (Kousteni et al., 2001). Furthermore, E2-treatment of COS-1 cells expressing ERα constructs targeted either to the nucleus, cytosol or plasma membrane demonstrated that only the membrane-associated ERα was capable of inducing Erk1/2 phosphorylation (Zhang et al., 2002). This E2 response occurred without increasing ERE promoter activation, and therefore, occurred independently of transcription.

As ERα does not contain a transmembrane domain, the ability of this receptor to localize to the plasma membrane is due to its association with membrane-bound proteins (Watson and Gametchu, 2003). ERα is found at the cellular membrane within calveolae/lipid rafts through direct association with caveolin-1 (Evinger and Levin, 2005). Binding is facilitated by palmitoylation on the cysteine447 residue in the E domain of human ERα. Replacement of Cys447 with an alanine (C447A) impairs human ERα palmitoylation and E2-induced Erk1/2 phosphorylation when these mutants are transfected in ER-null HeLa cells (Acconcia et al., 2004). Nuclear localization is also abrogated in mice harbouring a point mutation on Cys451 (the mouse equivalent of the human Cys447), where the cysteine residue was replaced with an alanine (C451A) (Adlanmerini et al., 2014). This mutation also highlighted the physiological importance of non-genomic E2 signalling, as critical arterial effects mediated by E2, such as stimulation of vasodilation and the promotion of endothelial repair, as well as female fertility, were compromised in these animals.

Several studies have sought to determine whether membrane-associated ERα represent a novel form of ERα, or if they are classic signalling ERα located outside of the nucleus. Breast
cancer MCF-7 cells that lack ERα (Márquez et al., 2006) or endothelial cells from combined ERα/ERβ-null mice (Razandi et al., 2004) do not express ERα localized at the plasma membrane. Furthermore, expression of ERα and ERβ in ER-null chinese hamster ovarian (CHO) cells resulted in both nuclear and membrane-localized functional pools of E2-binding ERα and ERβ (Razandi et al., 1999). In contrast to wild type endothelial cells, stimulation of endothelial cells lacking ERα/ERβ with E2 failed to activate cAMP, Erk1/2 or P13K, indicating that all functional ERs are absent in these cells (Pedram et al., 2006). This group also subjected subcellular ER pools from MCF-7 cells to tandem-array mass spectrometry and identified that the ERα located to the membrane and the ERα located in the nucleus arise from the same gene.

Rapid and transient activation of the MAPK pathway in response to E2-stimulation has been observed in a multitude of cellular contexts (Bi et al., 2000; Di Domenico et al., 1996; Endoh et al., 1997; Migliaccio et al., 1998, 1996; Neugarten et al., 1999). Magliaccio et al. demonstrated that E2-activation of MAPK is mediated through ERα, as transfection of human ERα cDNA is required for E2-dependent Erk1/2 phosphorylation is ER-null COS-1 cells (Migliaccio et al., 1998, 1996). In addition, this response is inhibited in ERα-positive MCF-7 cells and ERα-expressing COS-1 cells challenged with the anti-estrogen ICI 164 384. Membrane-association of ERα is also required, as MAPK activation is diminished in MCF-7 cells harbouring a point mutation in ERα preventing its association to the plasma membrane (Razandi et al., 2003). A transgenic mouse lacking all cellular ERα except a knock-in of the E domain targeted to the plasma membrane (membrane only ER (MOER) mouse) was generated, and demonstrated that while female reproductive tract and mammary gland development require nuclear ERα activity, membrane-localized ERα is necessary and sufficient for rapid signal transduction (Pedram et al., 2009). Here, Erk1/2 activation was comparable in the wild-type and MOER mouse livers, yet completely absent in mice lacking total ERα (αERKO).

Ligand binding mediates rapid, transient estrogen receptor cross-talk with several mitogenic pathways, leading to estrogen receptor phosphorylation at multiple sites by kinases, including MAPK (Figure 2) (for review, see (Ward and Weigel, 2009)). MAPK directly phosphorylates ERα
within the AF-1 domain at Ser118 (Bunone et al., 1996; Chen et al., 2002; Joel et al., 1998b; Kato et al., 1995), or indirectly on Ser167 through pp90RSK in response to E2, and these phosphorylation events result in ERE binding and subsequent transactivation of downstream elements. Furthermore, these residues also appear to be involved in the recruitment of transcription co-activator complexes at the promoters of target genes (Duplessis et al., 2011).

2.3 Reciprocal Interactions of c-src and ERα

C-src is a non-receptor tyrosine kinase involved in the regulation of cell proliferation and survival, and is frequently activated in human cancers (for review, see (Wheeler et al., 2009)). Initially, a direct interaction between c-src and ERα was shown using lysates prepared from E2-treated MCF-7 cells applied to a c-src-Sepharose column (Migliaccio et al., 1996). Subsequently, it was reported that c-src co-immunoprecipitates with ERα in E2-treated MCF-7 cells and LNCaP prostate cancer cells (Varricchio et al., 2007) and that this interaction was prevented in cells challenged with the anti-estrogen, ICI 182 780.

Migliaccio et al. showed that E2 stimulation of MCF-7 cells is accompanied with increased activity of Ras and the phosphorylation of c-src substrates, Shc and p190 (Migliaccio et al., 1998, 1996). Furthermore, Song et al. demonstrated that in addition to MAPK and Shc phosphorylation, E2 rapidly stimulated the association of Shc with ERα in MCF-7 cells (Song et al., 2002). Cells treated with PP2 or ICI 182 780, inhibitors of c-src and ERα, respectively, as well as expression of a dominant negative mutant of Shc showed that these factors act upstream of E2-mediated MAPK activation. Furthermore, a direct physical association of ERα with Shc was evidenced by protein pull-down assay.

A tyrosine residue in the AF-2 domain of ERα was identified that mediates receptor activation and interaction with c-src. Mutagenesis studies were completed in which the tyrosine 537 residue (Y537) in the human ERα was replaced with a phenylalanine (Y537F) and expressed in Sf9 cells (Arnold et al., 1997). While both mutants reacted with an anti-ER antibody, only the wild type ERα reacted with an anti-phosphotyrosine antibody, demonstrating that Y537 is the
only tyrosine residue phosphorylated on the human ERα (Figure 2). Subsequent studies showed that c-src is responsible for phosphorylating this residue, and that this action plays an important role in E2-dependent transcriptional activity of ERα (Arnold et al., 1997, 1995; Weis et al., 1996). Therefore, a model has been proposed in which reciprocal activation of ERα and c-src mediate rapid signal transduction and target gene transcription. E2-ERα rapidly and transiently activates c-src, which then activates downstream signalling cascades, such as Ras and MAPK (Migliaccio et al., 1998, 1996). Furthermore, The SH2-domain of c-src interacts directly with ERα at Y537 (Arnold et al., 1995), which rapidly induces signal transduction pathways involving Erk1/2 and P13K (Varricchio et al., 2007), as well as stimulating ERα target gene transcription (Sun et al., 2012).

E2 binding to ERα not only activates ERα transactivation, but also leads to rapid ubiquitin-dependent ERα proteolysis (Nawaz et al., 1999). E2 binding stimulates ERα phosphorylation at Y537 by c-src in MCF-7 cells, and this phosphorylation not only increases ERα binding to the E3 ubiquitin ligase, E6-AP, but also triggers ERα ubiquitylation (Sun et al., 2012). In contrast, the ERα harbouring Y537F failed to undergo E2-activated proteolysis. In addition, E6-AP knockout animals show increased mammary gland ER levels and mammary hyperplasia, which suggests that E6-AP also regulates ER levels in vivo (Ramamoorthy et al., 2010). Treatment of MCF-7 cells with c-src inhibitors or expression of c-src-targeted siRNA reduced E2-activated ubiquitylation and loss of cellular ERα (Sun et al., 2012). Furthermore, following E2-stimulation, the ERα/E6-AP complex is recruited to target gene promoters and enhances ERα transcriptional activity. Therefore, it is suggested that E2-mediated ERα phosphorylation at Y537 is involved in the regulation of both receptor stability and transcriptional activity.

3 HEDGEHOG SIGNALLING

The Hedgehog (Hh)-signalling pathway is an evolutionarily conserved cascade that represents one of the fundamental mechanisms required for the embryonic patterning and postnatal development of a myriad of tissues (for review, see (Ingham and McMahon, 2001)). Through
its involvement in regulating cell proliferation, differentiation and morphogenesis, Hh-signalling plays fundamental roles in controlling a range of processes, including left-right asymmetry (Tsiailris and McMahon, 2009), bone formation (Chuang and McMahon, 1999; Maeda et al., 2007), limb patterning (Chiang et al., 1996; Lee et al., 2001; Li et al., 2014; Zhulyn et al., 2014), and cell fate in the neural tube and brain (Chiang et al., 1996). Abrogated Hh-signalling can result in a range of birth defects including holoprosencephaly (Belloni et al., 1996; Roessler et al., 2009), while inappropriate pathway activity has been linked to human malignancies, such as basal cell carcinoma (Gailani and Bale, 1997; Gailani et al., 1996) and medulloblastoma (Berman et al., 2002; Oliver et al., 2005; Rudin et al., 2009).

3.1 Canonical Hedgehog Signalling

3.1.1 Ligand Synthesis

Mammals possess three Hedgehog ligands - Sonic Hedgehog (Shh), Desert Hedgehog (Dhh) and Indian Hedgehog (Ihh) - that mediate distinct processes during development (for review, see (Varjosalu and Taipale, 2008)). All Hh-ligands are synthesized as 45 kDa pro-proteins that, following translation, are transported to the endoplasmic reticulum (ER) where they undergo autoproteolytic cleavage to produce a 19 kDa N-terminal peptide and a 25 kDa C-terminal peptide (Bumcor et al., 1995; Lee et al., 1994). During autoproteolysis, the C-terminal fragment of the full-length Hh protein mediates the attachment of a cholesterol to the C-terminus of the N-terminal peptide (Porter et al., 1996a, 1996b). This peptide, termed N-Shh, is further modified by the addition of an amine-linked palmitic acid group to its most N-terminal cysteine (Chamoun et al., 2001; Pepinsky et al., 1998). The N-Shh product is the active species and participates in both local and long-range signalling (Martí et al., 1995; Porter et al., 1995). The significance of this autoproteolytic cleavage is emphasized by the fact that the development of holoprosencephaly is associated with mutations in Shh that disrupt its proper processing and formation of the N-Shh peptide (Maity et al., 2005; Roessler et al., 2009; Traiffort et al., 2004).
3.1.2 Ligand Secretion

Normal development of embryonic structures requires cellular proliferation and differentiation that is tightly regulated by secreted protein signals (Ulloa and Briscoe, 2007). Cells respond to specific concentrations of these signals to produce arrangements of different cell types that are required for the formation of the mature tissue (Freeman, 2000; Kutejova et al., 2009). N-Shh controls the development of a multitude of tissues in this fashion (for review, see (Ingham and McMahon, 2001)). Therefore, mechanisms that influence the distribution and concentration of N-Shh, such as those mediating the release (Callejo et al., 2011; Creanga et al., 2012) and sequestration (Chuang and McMahon, 1999; Goodrich et al., 1996) of signal, are important for driving the cellular responses involved in embryonic patterning. Despite dual lipid-modification, which firmly anchors N-Shh to the membrane of producing cells (Bumcrot et al., 1995; Peters et al., 2004), N-Shh signal can elicit distal responses in cells not immediately adjacent to Hh-ligand-producing sites (Basler and Struhl, 1994; Li et al., 2006; Strigini and Cohen, 1997; Wang et al., 2000). For example, target gene expression is induced in cells that are 5-10 cellular diameters away from Hh signal-producing cells in the wing imaginal disk of Drosophila (Basler and Struhl, 1994; Strigini and Cohen, 1997). The range of Hh signalling is even greater in vertebrate embryos, where N-Shh induces Ptc1 and Gli3 transcription across the limb bud, which encompasses many cell diameters (Goodrich et al., 1996; Li et al., 2006; Wang et al., 2000).

Dispatched (Disp) is a multi-spanning membrane protein required for the release of N-Shh from the lipid membrane in Drosophila (Burke et al., 1999). Loss of Disp causes accumulation of N-Shh in producing cells and consequently, loss of all long-rang N-Shh-elicited responses (Burke et al., 1999). Two murine homologues of Disp have been identified, and Disp1 in particular has been found to be essential for N-Shh signalling activity (Kawakami et al., 2002; Ma et al., 2002). The importance of this twelve-pass transmembrane protein was highlighted by the embryonic lethality of mice lacking Disp1 (Caspary et al., 2002; Kawakami et al., 2002; Ma et al., 2002). Interestingly, a N-Shh peptide lacking the added cholesterol moiety at its C-terminus is capable of eliciting ectopic Hh-pathway activation and spreads farther from the site of synthesis compared to
the wild type cholesterol-modified peptide (Li et al., 2006). In contrast, palmitoylation of N-Shh appears to promote its mobilization, as loss of palmitoylation diminishes the range of N-Shh activity (Chen et al., 2004).

More comprehensive molecular analysis has determined that N-Shh release is mediated by Disp1 acting in conjunction with a second protein, Scube2 (signal sequence, cubulin domain, epidermal growth factor-related), a secreted glycoprotein (Creanga et al., 2012; Tukachinsky et al., 2012). It has been shown through cross-link assays that Disp1 and Scube2 both associate with the cholesterol moiety of N-Shh at different locations (Tukachinsky et al., 2012), and together facilitate the release of N-Shh (Creanga et al., 2012; Tukachinsky et al., 2012).

3.1.3 Ligand Reception

Another important parameter regulating Hh-signal distribution is via sequestration by various transmembrane proteins. Feedback mechanisms have evolved to aid in the establishment of effective Hh signalling gradients within target tissues through the expression of cell surface proteins that bind to and limit the diffusion of Hh peptides (Holtz et al., 2013). Ptch1 is a transmembrane receptor that plays a pivotal role in the reception and transduction of Hh signals (Goodrich et al., 1996). This twelve-pass transmembrane receptor is expressed in Hh responding cells and binds all three mammalian Hh ligands with high affinity (Marigo et al., 1996; Stone et al., 1996). A negative regulator of the Hh-pathway, and a transcriptional target itself, \textit{Ptch1} expression is induced upon activation of the Hh-pathway (Bai et al., 2002; Goodrich et al., 1996). Through this negative feedback loop, Hh-signalling attenuates its own activity by increasing the amount of Ptch1 on the cell surface of Hh-responsive cells (Chen and Struhl, 1996; Jeong and McMahon, 2005).

Another Hh-binding protein, Hedgehog interacting protein-1 (Hip1), was identified in vertebrates and binds to all three mammalian Hh ligands with affinities similar to that of Ptch1 (Chuang and McMahon, 1999). Like Ptch1, Hip1 expression is induced by Hh signalling and participates in ligand-dependent negative feedback control (Chuang et al., 2003; Jeong and McMahon, 2005). While ectopic expression of Hip1 in transgenic animals impairs Hh activity
(Chuang and McMahon, 1999; Treier et al., 2001), loss of functional Hip1 results in exaggerated Hh signalling (Chuang et al., 2003). While no defects in limb bud or neural tube patterning were observed in mice lacking Hip1, disrupted lung branching and endochondral skeleton development was reported in these animals, which may be explained by functional compensation by Ptch1 (Chuang et al., 2003). These results suggest that not only can Ptch1 and Hip1 be functionally redundant, but the requirement of Hip1 for proper morphogenesis is tissue-specific. Evidence supporting functional redundancy of these two Hh receptors was illustrated by the earlier lethality (around E12.5) and more severe lung and pancreas defects in Hip1\(^{-/-}\) embryos also lacking one copy of Ptch1 (Hip1\(^{-/-}\);Ptch1\(^{+/+}\)) compared to Hip\(^{-/-}\) embryos (Chuang et al., 2003; Kawahira et al., 2003). However, while Hip1\(^{-/-}\) mice complete embryogenesis and only die shortly after birth (Chuang et al., 2003), Ptch1\(^{-/-}\) embryos die by E9.5 (Goodrich et al., 1997), revealing the distinct requirements for Ptch1 and Hip1 function during embryogenesis.

3.1.4 Ligand Signal Transduction

Cellular response to Hh signalling is mediated through the binding of Hh ligands to the system's receptor, Ptch1 (Goodrich et al., 1996; Marigo et al., 1996; Stone et al., 1996). Although there are two mammalian homologues of Ptch—Ptch1 and Ptch2, it is generally accepted that most signalling involves Ptch1, as mice that are homozygous for a Ptch2 mutation appear relatively normal (Nieuwenhuis et al., 2006). Ptch1 is required for suppressing downstream Hh signalling in the absence of ligand, as mice lacking Ptch1 exhibit complete ventralization of the neural tube, as well as embryonic lethality at E9.5 (Goodrich et al., 1997). In the absence of ligand binding, this twelve-pass transmembrane receptor constitutively represses Hh target gene expression by indirectly suppressing Smoothened (Smo) (Figure 3) (Denef et al., 2000; Ingham et al., 2000).

The predicted topology of Ptch1 is similar to that of bacterial resistance, nodulation, and disease (RND) transporters, which are multi-pass transmembrane proton efflux pumps associated with the transport of heavy metals and hydrophobic compounds (Tseng et al., 1999). Ptch1 also shows homology with eukaryotic multi-pass transmembrane proteins involved in cholesterol
transport by the sterol sensing domain (SSD), which spans the second to fifth transmembrane helices (Chang et al., 2006; Tseng et al., 1999). It has therefore been suggested that Ptch1 may regulate Smo activity through the release of cholesterol derivative intermediaries. This is supported by the observation that both pharmacological depletion and genetic loss of cholesterol biosynthesis inhibited the ability of fibroblasts to respond to Shh stimulation (Cooper et al., 2003). Furthermore, stimulation of fibroblasts with select oxysterols induced ciliary localization and Hh target gene activation (Dwyer et al., 2007).

Smo is a seven-pass transmembrane protein that belongs to the superfamily of G-protein-coupled receptors (GPCR), and is responsible for the transduction of Hh-signalling in both *Drosophila* (Alcedo et al., 1996; van den Heuvel and Ingham, 1996) and vertebrates (Chen et al., 2001; Zhang et al., 2001). Smo activity is critical for normal embryonic development, demonstrated by the severe developmental defects observed in Smo<sup>−/−</sup> mice, which resemble Shh<sup>−/−</sup> Ihh<sup>−/−</sup> double mutants. These studies indicate that loss of Smo activity results in complete loss of Hh-signal transduction (Zhang et al., 2001).

**Figure 3. Canonical Hh signalling pathway.** (A) In the absence of Hh-binding, Ptch1 inhibits Smo from entering the primary cilium. Proteolytic cleavage transforms Gli2/Gli3 into transcriptional repressors, which inhibit transcription of target genes. (B) Binding of Hh results in internalization of Ptch1 and subsequent translocation of Smo to the primary cilium. Gli2/Gli3 remain in the full length-activator form and translocate to the nucleus to activate transcription of target genes, such as Ptch1 and Gli1.

Transcription of Hh target genes is regulated by the Gli-family of transcription factors (Ruppert et al., 1988). Three members of this family have been identified in vertebrates: Gli1, Gli2 and Gli3 (Hui et al., 1994), that function as either transcriptional activators or repressors (Bai et al.,
Gli1 and Gli2 function primarily as transcriptional activators, although Gli2 does possess weak repressing capabilities (Aza-Blanc et al., 2000; Bai and Joyner, 2001; Sheng et al., 2002). In contrast, Gli3 functions primarily as a transcriptional repressor, but does also possess weak activating capabilities (Sasaki et al., 1999). These alternative weak activities tend to become apparent only in the absence of the other Gli family member (Bai et al., 2004; Buttitta et al., 2003; Motoyama et al., 2003). While animals lacking either Gli1 or Gli2 have normal digit patterning (Park et al., 2000), suggesting functional overlap between these two transcription factors, loss of one or both alleles of Gli3 is sufficient to produce severe polydactyly and ectopic expression of Shh target genes (Hui and Joyner, 1993; Schimmang et al., 1992). These phenotypes are indicative of constitutive Hh-signalling activity and highlight the importance of Gli3-mediated pathway repression. The ability of Gli proteins to act as transcriptional activators depends on the presence of a C-terminal activation domain. Strategic proteolytic cleavage of the full length Gli (Gli-FL) protein results in the formation of the N-terminal Gli repressor (Gli-R) (Sasaki et al., 1999). Gli1 lacks this N-terminal domain and can therefore only function as a transcriptional activator (Dai et al., 1999). Also, as Gli1−/− mice are viable and maintain normal development, Gli1 appears to be dispensable for mammalian development (Bai et al., 2002; Hynes et al., 1997; Lee et al., 1997; Park et al., 2000).

In addition to Ptch1, several other regulatory factors are involved in mediating repression of mammalian Hh-signalling in the absence of ligand. Suppressor of Fused (Sufu) directly influences the activity of Gli transcription factors. Through binding to the full length Gli1, Gli2 and Gli3, Sufu sequesters these proteins, and prevents their nuclear translocation and activation (Humke et al., 2010; Kogerman et al., 1999; Tukachinsky et al., 2010). Murine knockout models revealed that Sufu−/− mice exhibit embryonic lethality at E9.5 with significantly reduced levels of both full-length and repressor forms of Gli, as well as developmental abnormalities indicative of aberrant Hh activation that resemble loss of Ptch1 (Cooper et al., 2005; Svärd et al., 2006). Sufu is also required for Gli-R formation in vertebrates. Sufu promotes the phosphorylation in the C-terminus of Gli-FL by protein kinase A (PKA), priming Gli-FL for further phosphorylation by glycogen phosphorylase.
synthase kinase 3β (GSK3β) and casein kinase 1α (CK1α) (Kise et al., 2009; Tempé et al., 2006). This phosphorylation results in the ubiquitination and degradation of the Gli-FL C-terminal peptide to generate Gli-R (Bhatia et al., 2006; Kise et al., 2009; Tempé et al., 2006; Wang and Li, 2006). While Gli3 is preferentially processed to its repressor form, the processing of Gli2 is much less efficient (Bhatia et al., 2006; Pan et al., 2006). The increased efficiency of Gli3 processing in comparison to that of Gli2 is due in large part to a C-terminal domain, termed the processing determinant domain (PDD), that determines partial or complete proteosomal degradation of the C-terminus of the protein (Pan and Wang, 2007).

In addition to Sufu, the kinesin-4 family member, Kif7, also appears to be required for optimal Gli processing in the absence of ligand (Cheung et al., 2009; Endoh-Yamagami et al., 2009; Liem et al., 2009; Tay et al., 2005). Mice deficient in Kif7 have increased levels of Gli-FL and decreased levels of Gli-R, and also exhibit features of pathway derepression, such as polydactyly (Cheung et al., 2009; Endoh-Yamagami et al., 2009; Liem et al., 2009). Although the mechanism by which Kif7 promotes Gli processing remains unclear, one possibility is that Kif7 recruits PKA, GSK3β, and CK1α to phosphorylate Gli-FL. PKA, has also been found to be an essential negative regulator of mammalian Hh signalling (Huang et al., 2002; Wang et al., 2000). As mentioned previously, this protein initiates the phosphorylation of Gli-FL required for the production of functional Gli-R. PKA null mice display ectopic Hh-pathway activation, resulting in severe neural tube defects and embryonic lethality at E9, similar what is observed in Ptch1−/− and Sufu−− animals (Tuson et al., 2011).

Although Hh signal transduction is mediated by ligand binding to Ptch1, Hh reception is modulated by additional Hh-binding proteins existing on the plasma membrane of Hh responsive cells (Allen et al., 2011). Cdo (CAM-related/downregulated by oncogenes), Boc (brother of Cdo) (Tenzen et al., 2006; Yao et al., 2006) and Gas-1 (growth arrest-specific 1) (Martinelli and Fan, 2007) are cell-surface proteins that function with Ptch1 as co-receptors for Hh proteins and facilitate Hh-signal transduction. Genetic studies in Drosophila and mice suggest that unlike Ptch1, these co-receptors, function to positively regulate Hh signalling (Allen et al., 2007; Cole and Krauss,
2003; Martinelli and Fan, 2007; Tenzen et al., 2006; Yao et al., 2006; Zhang et al., 2006). When bound to these receptors Hh signalling is enhanced in what appears to be a synergistic interaction with Ptch1 (Yao et al., 2006).

3.1.3 The Primary Cilium

The primary cilium is a microtubule-based organelle involved in the signal transduction response to the surrounding cellular environment (Goetz and Anderson, 2010). The realization that vertebrate Hh-signalling depends on the primary cilium came from a phenotype-based screen for mutations that alter patterning of the mouse embryo (Huangfu et al., 2003). This screen identified several mutants displaying morphological and patterning phenotypes consistent with altered Hh signalling. The mutations analyzed in these initial studies resulted either in structural defects of the primary cilia, or the complete absence of the primary cilia, and produced abnormalities characteristic of reduced Hh signalling. Specifically, embryos harbouring mutations that disrupt intraflagellar transport (IFT) proteins, which transports proteins up and down along the axoneme (the central microtubule-based structure in the cilia), die around E10.5-11.5, and display defective neural tube phenotypes characteristic of mice lacking Shh (Caspary et al., 2002; Ma et al., 2002). Furthermore, IFT proteins are required Gli2/3 processing and trafficking and as a result, IFT mutants lack all responses to Hh ligands (Haycraft et al., 2005; Huangfu and Anderson, 2005; Liu et al., 2005).

Hedgehog responsive cells are ciliated and all key components of the Hh pathway are found within the cilia (Figure 3) (Corbit et al., 2005; Haycraft et al., 2005; Merchant et al., 2005; Rohatgi et al., 2007). In the absence of ligand binding, Ptch1 is localized at the base of the cilium, where it maintains Smo in an inactive conformation outside of the cilium in the adjacent membrane. Binding of Hh-ligand to Ptch1 triggers the reciprocal trafficking of Ptch1 and Smo (Denef et al., 2000). While Ptch1 translocates out of the cilium and is subsequently internalized, Smo is trafficked into, and accumulates in the cilium. Ciliary localization of Smo correlates with Hh-pathway activation, as both Smo agonists and an oncogenic mutation of Smo promotes its accumulation in the cilium.
(Corbit et al., 2005; Rohatgi et al., 2007). Phosphorylation of the Smo C-terminus by protein kinase A (PKA) and casein kinase I (CKI) is critical for Hh signal transduction in Drosophila (Fan et al., 2012; Su et al., 2011). However, the sequence of vertebrate Smo C-terminus is significantly different from that of Drosophila and lacks the C-terminal PKA phosphorylation sites (Apionishev et al., 2005; Jia et al., 2004; Zhao et al., 2007a, 2007b). Molecular analysis has determined that two kinases, G-protein coupled receptor kinase 2 (GRK2) and CKIα, phosphorylate the C-terminus of Smo in vertebrates and mediates its conformational change into an active state in response to Hh ligand (Chen et al., 2011; Zhao et al., 2007a).

In the absence of ligand, PKA and Kif7, are located at the base of the primary cilium, where they promote proteolytic processing of Gli3 by the proteasome into Gli-R (Endoh-Yamagami et al., 2009; Tuson et al., 2011). In addition, Sufu and PKA stabilize Gli proteins and inhibit the transcriptional activity of Gli2 by sequestration at the base of the cilium (Tuson et al., 2011). The activation of Smo abrogates PKA function and promotes the movement of Sufu–Gli2/3 complexes to the tip of the cilia (Tukachinsky et al., 2010), dissociating this complex in the process. Kif7 may also promote Sufu-Gli disassembly, as it localizes to the cilium in response to Hh and interacts with overexpressed Smo in tissue culture cells (Endoh-Yamagami et al., 2009). This dissociation allows Gli2 to translocate to the nucleus, where it is converted to a transcriptional activator (Gli-A) and drives transcription of target genes (Humke et al., 2010; Tukachinsky et al., 2010).

3.2 Non-Canonical Hedgehog Signalling

Growing evidence suggests the existence of additional signalling cascades activated by Hh-ligands but deviate from the ‘canonical’ paradigm (Figure 4) (for review, see (Jenkins, 2009)). Several ‘non-canonical’ signalling scenarios have been suggested, including signalling events involving Hh pathway components operating independently of Smo or Gli-mediated transcription.

The identification of a direct interaction between Ptch1 and cyclin B1, a critical regulator of mitotic cell division, first suggesting a role for Ptch1 in the regulation of a cell cycle G2/M checkpoint (Figure 4) (Barnes et al., 2001). In this study, the interaction of Ptch1 and cyclin B1
was identified in a yeast two-hybrid screen using a phospho-mimetic cyclin B1 as bait. Subsequent co-immunoprecipitation assays revealed that Ptch1, through its large intracellular loop, binds to phosphorylated cyclin B1. Cellular fractionation studies demonstrated that the interaction of cyclin B1 with Ptch1 occurs predominantly at the cellular membrane. The addition of Shh, however, disrupts this interaction and allows cyclin B1 to localize to the nucleus. Collectively these data support a model in which a cyclin B1-Ptch1 complex inhibits cellular growth. Specifically, Ptch1 sequesters phosphorylated cyclin B1 in the cytoplasm near the cellular membrane in the absence of ligand. Upon activation, through stimulation with Shh, this interaction is broken, allowing cyclin B1 to translocate to the nucleus and promote cell cycle progression.

A C-terminal Ptch1 polymorphism has been identified in the FVB strain of mice, that effects the ability of Ptch1 to complex the molecular chaperone, Tid1 (Figure 4) (Wakabayashi et al., 2007). Tid1 has been implicated in signalling pathways involving Ras (Taruolina et al., 2004; Trentin et al., 2001), Smads (Torregroza and Evans, 2006), as well as in the control of apoptosis (Edwards and Münger, 2004). This polymorphism explains why FVB mice are highly susceptible to the development of skin squamous carcinomas (SCCs) induced by an activated HRas oncogene, while mice of the C57BL/6 strain are resistant (Wakabayashi et al., 2007).

Previous work from our lab showed that the C-terminal region of Ptch1 contains highly conserved sequences that encode consensus binding sites for Class I and Class II SH3-domains, as well as WW-domains (Chang et al., 2010). GST-pull down assays and co-immunoprecipitation revealed that the isolated full length C-terminus of Ptch1 (Ptch1\textsuperscript{C-term}) binds to several SH3-domain containing factors, including Grb2, c-src, and the PI3K regulatory subunit p85β (Chang et al., 2010). Furthermore, activation of c-src by N-Shh stimulation was observed in Shh-Light II fibroblasts, as well as in the mammary epithelial cell line, MCF10A (Chang et al., 2012). A transient 2-fold increase in activated c-src was observed for these cell lines following N-Shh stimulation. This activation, however, was inhibited in cells treated with the N-Shh neutralizing monoclonal antibody, 5E1 (Ericson et al., 1996; Maun et al., 2010). Since MCF10A cells have no detectable expression of Smo (Chang et al., 2010; Mukherjee et al., 2006; Zhang et al., 2009), we
suggested that activation of c-src occurs through pathways operating through Ptch1 independent of its regulation of Smo activity.

More recently, our lab has published results showing that activation of c-src and its interaction with Ptch1 is N-Shh dependent (Figure 4) (Harvey et al., 2014). Transfected Ptch1 co-immunoprecipitates with endogenous c-src in the absence of Hh signalling in MCF10A cells. This association was not detected in cells stimulated with N-Shh. Furthermore, increased activation of c-src, evident through increased detection of c-src phosphorylated on Y416, was observed in cells stimulated with N-Shh. Subsequent experiments revealed that constitutive activation of c-src was observed in primary mouse fibroblasts harbouring the mesenchymal dysplasia (mes) allele of Ptch1, which results in a truncated form of Ptch1 that is missing the majority of the C-terminus (Makino et al., 2001). This activation was not observed in cells harbouring wild type Ptch1 (Harvey et al., 2014). Together, we reported that the activation of c-src, as well as its interaction with the C-terminus of Ptch1 is dependent on N-Shh, and that this signalling cascade operates independently of Smo.

In addition to c-src, our lab has demonstrated that N-Shh stimulation activates Erk1/2 (Figure 4). Experiments in cultured fibroblast and epithelial cell lines demonstrated that stimulation with N-Shh results in MEK-dependent activation of Erk1/2 (Chang et al., 2010). This activation was
observed in Smo-deficient MCF10A cells (Mukherjee et al., 2006; Zhang et al., 2009), as well as in Shh Light II fibroblasts and the human mammary epithelial cell line, HMEC, in the presence of the Smo inhibitor, cyclopamine (Incardona et al., 1998). These results suggest that N-Shh dependent activation of Erk1/2 occurs independently of Smo activity.

Recently, a role for Hh signalling in human hepatocellular carcinoma (HCC) invasion and metastasis has been identified (Lu et al., 2012). Several studies have shown that Hh-signalling upregulates cell migration and invasion in human gliomas and in pancreatic cancer by increasing the expression of matrix metalloproteinase-9 (MMP-9) (Onishi et al., 2011; Wang et al., 2010), and it was hypothesized that this expression of MMP-9 was dependent on Hh-mediated activation of Erk1/2 (Lu et al., 2012). This group identified a significant correlation between Gli1, p-Erk1/2 and MMP-9 expression and pathological grade and metastasis of HCC tumours. Treatment of N-Shh significantly increased invasion and migration of Bel-7402 cells. This invasion was inhibited in cells treated with the MEK inhibitors, U0126 and PD98059. In addition, stimulation of Bel-7402 cells with N-Shh significantly increased expression of p-Erk/12 and MMP-9. However, in contrast to what our lab has reported (Chang et al., 2010; Harvey et al., 2014), this expression was inhibited in the presence of cyclopamine (Lu et al., 2012).

Another group reported N-Shh dependent activation of Akt and Erk1/2 in the myoblast C2 cell line (Elia et al., 2007). In these cells, the addition of cyclopamine inhibited this Shh-induced phosphorylation, suggesting that Shh effects on Akt and Erk1/2 are Smo-mediated. However, N-Shh did not have any inductive effect on the phosphorylation of Akt or Erk1/2 in the human prostate cancer cell line, PC3, which is known to show canonical Hh activation in response to N-Shh stimulation (Mimeault et al., 2006). This demonstrates that Shh effects on signalling pathways is cell-type specific, which explains the differential requirements of Smo activity in N-Shh mediated Erk1/2 activation in different cellular contexts.

4 MAMMARY GLAND DEVELOPMENT

Like other skin appendages, proper development of the mammary gland requires extensive
epithelial-mesenchymal interactions (for review, see (Wiseman and Werb, 2002)). Although morphogenesis begins embryonically, important aspects of its development occurs in the postnatal animal. This organ does not remain quiescent. Recurrent estrous cycles in adulthood trigger extensive side branching in response to hormone stimulation. Also, with each pregnancy, the mammary gland also undergoes a cyclic regimen of proliferation and differentiation, producing extensive ductal branching and milk producing alveolar cells, followed by post-lactational regression and apoptosis with weaning.

A number of cell types make up the mammary gland (for review, see (Richert et al., 2000)). Two main types of epithelium form the extensive ductal network of the gland: (1) luminal epithelial cells form the ducts and the secretory alveoli, and (2) basal cells consist of mainly myoepithelial cells. Together, these two types of epithelium form the bi-layered ductal structure that is embedded within the fatty stroma. These ducts carry milk from the milk producing alveolar cells to the nipple. There are also adipocytes, which make up the fat pad in which the ductal network is embedded; vascular endothelial cells that make up blood vessels; stromal cells, including fibroblasts; and a variety of immune cells.

There are three main stages of mammary gland development in rodents and in humans: embryonic, pubescent and adult (for review, see (Masias and Hinck, 2012)). In the murine embryo, following mesenchymal stimulation, five pairs of mammary buds sprout and branch to form a rudimentary ductal structure that embeds in the subdermal fat pad. At birth, five pairs of glands are present in the mouse, each comprising of a rudimentary ductal tree of 10-20 branches that occupies a minor portion of an overall empty fat pad. These mammary glands enter a period of relative quiescence, in which they showed isometric growth, keeping up with overall body development until the start of puberty (Howard, 2012). The focus of this work will be on the pubescent stage of mammary gland development.

At the onset of puberty (approximately three weeks of age), ovarian stimulation results in the formation of terminal end buds (TEBs) at the tips of the growing ducts (Figure B) (Ball, 1998). These bulbous, club-shaped structures are comprised of a single outer layer of highly proliferative
cap cells (Williams and Daniel, 1983) surrounding a multi-layered core of pre-luminal body cells. The body cells will differentiate into multiple layers of luminal epithelial cells (Daniel et al., 1995), and upon pregnancy, will differentiate further into milk-producing lobulo-alveolar cells (Gordon and Bernfield, 1980). The cap cells differentiate into the contractile myoepithelial cells that form the outer layer of the mature mammary duct that is surrounded by the basement membrane (Williams and Daniel, 1983). These cells lead the TEB in its invasion of the fat pad (Figure 5A). The lumen of mammary ducts is thought to form through cavitation, a process involving selective apoptosis of cells in the centre of a thickened cluster and the polarization of cells contacting the basement membrane, resulting in a parent epithelial tube. This has been seen in vivo, as well as mammary acini in 3D culture models (Debnath et al., 2002). In accordance to this hypothesis, apoptosis has

Figure 5. Pubescent mammary gland development. (A) Schematic of mammary gland development showing ductal elongation during puberty. (B) Terminal end bud (TEB). Immunohistochemistry staining probing cap cells with p63 surrounding body cells. (C) Whole mount of post-pubescent mammary gland #4.
been detected in body cells of developing tubes (Humphreys et al., 1996).

Secondary branches sprout laterally from the primary ducts until a tree-like pattern of ducts occupies the majority of the available fat pad. By approximately 10-12 weeks, at the end of puberty, the ducts reach the limits of the fat pad, the TEBs disappear and growth ceases (Figure 5C). Under cyclical ovarian hormone stimulation, short tertiary branches will form, temporarily filling the fat pad even more, but full milk-secreting alveolar cell differentiation occurs only under the influence of pregnancy hormones (Graham and Clarke, 1997). Rapid proliferation during pregnancy results in the formation of extensive tertiary branches and the formation of milk producing alveoli. Following lactation, removal of alveolar cells and excessive ducts is accomplished by involution and apoptosis (Li et al., 1997).

4.1 ERα Signalling in Mammary Gland Development

Manipulation of mammalian hormone signalling, either through gross endocrine ablation or the generation of gene targeted hormone receptor knockouts, have given insight into the hormonal requirements of the various stages of mammary gland development (for review, see (Rosen, 2012)). Since there have been no observations of overt mammary gland development before puberty in mice lacking various hormone receptors (Couse and Korach, 1999), it is believed that up until puberty the mammary gland develops in a hormone-independent fashion. In contrast, pituitary and ovarian hormones are absolutely essential for pubertal and adult mammary gland morphogenesis (Dupont et al., 2000; Mallepell et al., 2006).

The importance of E2 in mammary gland morphogenesis was illustrated by the rescue of development in ovariectomized mice, which do not exhibit ductal elongation, by administration of exogenous E2 (Daniel et al., 1987). However, due to the complexity in which hormones signal, it was difficult to determine to what extent E2 was effecting the mammary gland, and what was the result of a secondary stimulation of other organs and hormones. The direct effect of E2 on the mammary gland was then illustrated by the inhibition of mammary morphogenesis in mice with locally implanted antiestrogens (Daniel et al., 1987; Silberstein et al., 1994).
Mice lacking ERα (αERKO) display no development of ductal structures past the rudimentary tree that develops during embryogenesis (Mallepell et al., 2006). Since ERα is expressed both in the mammary epithelium and in the mammary stroma (Daniel et al., 1987), transplantation experiments were completed to determine which pool of ERα is responsible for the E2-driven ductal elongation during puberty (Mallepell et al., 2006). Wild type epithelium grafted onto the cleared fat pads of ERα−/− hosts grew and filled the fat pad. In contrast, ERα−/− epithelium grafted onto the fat pads of ER−/− hosts failed to grow, demonstrating that epithelial ERα is required for pubescent mammary gland morphogenesis. Similar transplantation experiments were also conducted to determine the role of stromal ERα in mammary gland biology. However, since wild type epithelium grew in ERα−/− fat pads, it was determined that stromal ERα is not involved in pubertal mammary gland development.

ERα-expressing epithelial and stromal cells do not proliferate (Clarke et al. 1997; Russo et al. 1999; Seagroves et al. 2000; Grimm et al. 2002). To determine if ERα drives cell cycle progression in a paracrine fashion, ERα−/− mammary epithelial cells were mixed with wild type mammary epithelial cells, and then grafted onto wild type hosts to reconstitute cleared fat pads (Mallepell et al., 2006). These studies showed that the ERα−/− epithelial cells proliferate in the presence of surrounding wild type epithelial cells, indicating that E2 can act in a paracrine fashion to elicit proliferation of adjacent ERα−/− cells.

4.2 Hedgehog Signalling and Mammary Gland Development

The Hh pathway is crucial for the patterning and growth of many vertebrate epidermal appendages that depend on epithelial-mesenchymal interactions (Chuong et al., 2000). An absolute requirement for Hh activity was identified in the development of hair (St-Jacques et al., 1998), teeth (Hardcastle et al., 1998) and feathers (Ting-Berreth and Chuong, 1996). As hair follicles and mammary glands share the requirement of several local signalling pathways (Andl et al., 2002; Chen et al., 2004; Hens and Wyssolmerski, 2005), it was hypothesized that the requirement of active Hh signalling for proper development would also be common among these tissues. However, despite their common origin, mammary glands differ from hair follicles and other epidermal
appendages in their requirement for Hh pathway repression rather than activation (Gallego et al., 2002).

Components of the Hh-signalling pathway, including all three Hh ligands (Gallego et al., 2002; Kouros-Mehr and Werb, 2006; Michno et al., 2003), downstream transcription factors, Gli2 and Gli3 (Hatsell and Cowin, 2006; Lewis et al., 2001), as well as Hh transcriptional target genes, Gli1 and Ptc1f1, have been described in the mammary gland (Gallego et al., 2002; McDermott et al., 2010). Specifically, Ptc1 has been reported in the body cells of the TEB, as well as in the differentiated ductal epithelium and in the periductal stroma (Lewis et al., 1999). Gli2 is expressed within the surrounding stroma of the TEBs (Lewis et al., 2001), and Gli3 has been found both in the epithelium and stroma (Hatsell and Cowin, 2006). However, as determined by lacZ-based transcriptional reporter assays, both Gli1 and Ptc1f1 expression levels are low, suggesting that Hh-signalling may be absent or repressed in mammary cells. Supporting this hypothesis were the observations that while embryos null for either Gli1 or Gli2 show no defects in mammary bud formation, constitutive activation of Gli1 or lack of Gli3 expression results in failed bud formation in the embryo (Hatsell and Cowin, 2006).

The role for Hh-signalling in the postnatal mammary gland remains largely undefined due, in part, to the embryonic lethality of mice lacking key components of the pathway (Goodrich et al., 1997; St-Jacques et al., 1999; Svärd et al., 2006; Zhang et al., 2001). Transplantation of Shh-null or Ihh-null mammary anlagen into cleared fat pads of wild type animals was completed to determine the involvement of these ligands in the adult mammary setting (Gallego et al., 2002). To ensure development was not a consequence of morphogenic activities of endogenous ligand expressed in surrounding tissues, Ihh-null or Shh-null anlagen were also transplanted under the renal capsule of wild type host mice. Both Shh-null and Ihh-null transplants developed normally, and showed no alteration in Ptc1f1 expression. Although functional redundancy between these ligands may account for the lack of mammary phenotype in these mutants, these experiments suggest that Ihh and Shh are dispensable for pubescent mammary gland development (Gallego et al., 2002; Michno et al., 2003).
Transgenic mice harbouring loss-of-function alleles for the Gli genes have been generated (Lewis et al., 2001; Park et al., 2000). Transplantation experiments of Gli2-null anlagen into cleared fat pads of wild type hosts revealed that stromal Gli2 is required for proper ductal patterning (Lewis et al., 2001). Although glands derived from Gli2-null embryos showed ductal outgrowths, ducts were frequently distended or irregularly shaped. Furthermore, constitutive expression of Gli1 in mammary epithelial cells under the control of the MMTV promoter lead to defects in alveolar network complexity, as well as an inability of these mice to lactate (Fiaschi et al., 2007). Hyperplastic lesions and tumour development was also observed in these animals.

Heterozygous loss of Ptch1, resulting in partial derepression of the pathway, lead to morphological abnormalities in TEBs and ducts in virgin mice (Lewis et al., 1999). While homozygous deletion of Ptch1 results in early embryonic lethality, heterozygotes survive. These animals developed defects of the mammary gland, including occluded ducts, which were reversible during pregnancy, which was thought to be the result of compensatory effects of enhanced Ihh expression that occurs during pregnancy. In addition, derepression of the Hh pathway in the mammary glands of mice harbouring an activated allele of Smo within the mammary luminal epithelium (MMTV-SmoM2) lead to abnormal and retained TEBs (Moraes et al., 2007). Furthermore, this group observed increased cellular proliferation and ductal dysplasia in these animals. More recently, this group determined high levels of Ptch2, Gli1 and Gli2 expression in SmoM2-expressing epithelial cells, which drove proliferation in adjacent cells (Visbal et al., 2011).

Cilia are found on luminal epithelial, myoepithelial, and stromal cells during early branching morphogenesis in pubescent mice. Once branching is complete, cilia mostly disappear and remain only on the myoepithelial and stromal cells in the adult mammary gland (McDermott et al., 2010). The mammary glands of mice harbouring IFT defects (Tg737orpk) show reduced ductal extensions and decreased secondary and tertiary branching, which is associated with ciliary dysfunction. In addition, this group observed increased canonical Wnt-signalling and decreased Hh-signalling in mammary epithelial cells in Tg737orpk mice, which has been associated with
decreased branching morphogenesis in other tissues (Dean et al., 2005; Jaskoll et al., 2004).

Using the *mesenchymal dysplasia (mes)* mouse (Makino et al., 2001), our lab, in collaboration with the lab of Dr. Michael Lewis, definitively showed that Hh signalling is absolutely required for proper morphogenesis of the postnatal mammary gland. The *mes* allele encodes a deletion in second-last exon of *Ptch1*, resulting in a truncated protein that replaces the last 220 a.a. with a random 68 a.a. polypeptide (Makino et al., 2001; Sweet et al., 1996). *Mes* homozygote mice display a wide range of developmental defects, including polydactyly on all feet, white belly spot, excess skin, increased musculature (specifically around the shoulder and hip areas) and a short, kinky tail (Makino et al., 2001). More importantly, we have determined that the mammary glands of these animals fail to develop (Chang et al., 2012; Moraes et al., 2009). In *mes* homozygotes, the fat pads of glands remain clear throughout puberty and remain in this primitive state, even at 24 weeks (Chang et al., 2012).

No activation of the Hh signalling pathway was evident in the epithelial or stromal compartments of the *mes* mammary glands that would be expected if the *mes* mutation abrogated *Ptch1*-mediated pathway suppression. Surprisingly, *Gli1* and *Ptch1* mRNA levels do not differ in the mammary glands between *mes* mice and wild type littermates. These data suggest that, in accordance to what has been previously shown in cultured Smo-deficient or Smo-inhibited fibroblast and epithelial cells (Chang et al., 2010; Harvey et al., 2014), Hh-ligands invoke signalling cascades through the C-terminus of *Ptch1* independently of Smo activity. The previously mentioned GST-pull down data (Chang et al., 2010) in conjunction with the observation that stimulation of cultured fibroblasts and mammary epithelial cells with N-Shh can activate c-src (Chang et al., 2012), as well as the requirement of c-src activity in mammary gland development (Kim et al., 2005), led to the hypothesis that c-src may function downstream of *Ptch1* in a signalling system that is abrogated in *mes* mice. To test this, a constitutively active allele of c-src (c-src<sup>A</sup>) was expressed in mice under the control of the mouse mammary tumour virus (MMTV) promoter on the *mes* background (Chang et al., 2012). Forced luminal epithelial expression of c-src<sup>A</sup> rescued blocked ductal morphogenesis in *mes* mice, however, with delayed kinetics. Expressing
the MMTV-ErbB2\textsuperscript{Act} transgene on the mes background did not rescue the block in mammary gland development, indicating that c-src is a specific downstream effector of Ptch1.

The block to mammary development in mes homozygotes seems to be a result of abrogated ovarian hormone signalling. While ovaries appear histologically normal in mes mice, mammary glands of mes homozygotes failed to develop following 4 weeks of injections with exogenous estrogen (Chang, unpublished observation). These results suggested that the mes mammary phenotype is not a result of lack of hormone production, but an inability of mammary epithelial cells to respond to ovarian stimuli. This abrogated E2 signalling suggests that ER\(\alpha\) may be driving ductal elongation during puberty by acting in a signalling cascade downstream of Ptch1. This model is supported by the significant decrease in the level of ER\(\alpha\) expressed in the epithelial and stromal compartments of the mammary glands in mes mice (Chang et al., 2012). The involvement of c-src in this signalling cascade was supported by the rescue of ER\(\alpha\) expression in mes mice expressing c-src\textsuperscript{Act}. Furthermore, increased levels of ER\(\alpha\) expression in mammary glands was observed in all animals, both mes and wild type, expressing c-src\textsuperscript{Act}. This requirement of c-src activation for ER\(\alpha\) expression in mammary gland development is consistent with the lack of ER\(\alpha\) activation in c-src-null mice (Kim et al., 2005).
Rationale

Work previously completed in our lab identified novel factors that bound to Ptch1 and revealed novel signalling pathways stimulated by Hh-ligands. We showed further the requirement for Hh-signalling in normal mammary morphogenesis using the mes mouse harbouring a mutation in the C-terminus of Ptch1. The rescue of mes mammary morphogenesis and induction of ERα expression by forced expression of a constitutively active c-src suggests that Hh-signalling in mammary epithelial cells involves the activities of c-src and ERα.

Hypothesis & Objectives

We hypothesis that ERα operates in a signalling cascade downstream of Hh that is required for postnatal mammary gland development.

Figure 6. ERα acts genetically downstream of Ptch1 in mammary gland development. Schematic of proposed signalling cascade involving Hh, c-src and ERα.

The objectives are as follows:

1. Determine if ERα acts genetically downstream of Ptch1 in vivo by expressing an ERα transgene in the mammary epithelial cells in the mes mice
   - Determine the effect of ERα expression on the proliferative state of the mammary gland
   - Determine whether epithelial ERα expression can facilitate stromal activation in the mammary gland
2. Determine the stimulatory effect of Hh-ligands on specific cell types in the mammary gland

- Identify canonical Hh-signalling in primary mammary epithelial cells and fibroblasts

- Elucidate the non-canonical Hh-signalling cascade involving Erk1/2 in mammary epithelial cells
Materials & Methods

Cell Culture

HEK 293 cells (a gift of Prof. S. Girardin) were cultured in DMEM with 10% FBS and 1% penicillin-streptomycin (invitrogen). Shh Light II fibroblasts (ATCC) were cultured in DMEM with 1% penicillin-streptomycin, 400μg/ml G418 (Gibco) and 0.14μg/ml Zeocin (Invitrogen).

Mice

The conditional ERα in mammary epithelium tissue (CERM) mice were a gift from Priscilla A. Furth; Georgetown University (Frech et al., 2005; Hruska et al., 2002). CERM mice harbour a transgene composed of the mouse mammary tumour virus long terminal repeat (MMTV-LTR) driving expression of the tetracycline responsive reverse transactivator (rtTa) (Frech et al., 2005; Gunther et al., 2002). They also harbour an independent transgene encoding murine flag-tagged ERα that is under the control of the tetracycline response operator (tetO) (Hruska et al., 2002). CERM mice were backcrossed onto C57Bl/6N (CharlesRiver) for >4 generations and finally crossed with C57Bl/6N mice heterozygous for the mesenchymal dysplasia (mes) allele of Ptch1 to produce compound homozygous mes/CERM mice.

For genotyping, tail DNA was extracted and 2μl DNA was amplified in a 25μl polymerase chain reaction using Taq DNA Polymerase (Thermo Scientific). Forward (F) and reverse (R) primer sequences were as follows:

Mes: (F) 5’-TCCAAGTGTCGTCGCCGGTTTG-3’,
(R) 5’-GTGGCTTCCACAATCACTTG-3’ (Makino et al., 2001);
Flag-ERα: (F) 5’-CGAGCTCGGTACCCGGGTCG-3’,
(R) 5′-GAACACAGTGGGCTTGCTGTTG-3’ (Miermont et al., 2010);
MMTV-rtTa: (F) 5’- ATCCGCACCCCTTGCTGCTTG-3’,
(R) 5’ GGCTATCAAACACACTGCCAC-3’ (Miermont et al., 2010).

Reaction conditions for MMTV-rtTa and mes were 60 seconds each for denaturation, annealing,
and extension for 32 cycles. The reaction conditions for Flag-ERα were 60 seconds denaturation, 90 seconds annealing, and 120 seconds extension for 35 cycles. The annealing temperatures for mes, Flag-ERα and MMTV-rtTa were 53°C, 57°C and 56°C, respectively.

Transgene expression was induced through long-term administration of doxycycline. Mice were supplied continuously with sterile filtered water containing 2mg/ml doxycycline (Gunther et al., 2002) and 5% sucrose (changed twice per week to prevent mold growth). Treatment began at the onset of puberty (three weeks of age) and continued until their endpoint.

**Whole Mount Analysis**

Whole mount analysis of mammary glands was performed as previously described (Chang et al., 2012; Rasmussen et al., 2000). Mammary glands #4 and #5 were isolated from euthanized female mice at varying ages and stretched over a microscope slide (Fisherbrand). Glands were fixed in Carnoy’s fixative (10% acetic acid, 30% chloroform, 60% ethanol) overnight at 4°C, and washed in 70%, 50% and 25% ethanol for 15 min each. The glands were rinsed in water for 5 min and then stained in Carmine alum overnight at room temperature. The glands were dehydrated in 70%, 95% and 100% ethanol for 15 min each, cleared in xylene for two 30 min changes, and then mounted with Permount (Fisherbrand).

**Immunofluorescence**

Mammary glands #4 and #5 were isolated together from euthanized female mice of varying ages. For Bromodeoxyuridine (BrdU) incorporation, 50µg/kg BrdU (Life Technologies) was administered to mice through subcutaneous injection 2hr before they were euthanized and glands harvested. Isolated mammary glands were fixed in 4% paraformaldehyde in PBS for 2hr at room temperature. Glands were then washed in 50% ethanol. Glands were embedded in paraffin and cut into 4 micron-thick sections (Toronto Centre of Phenogenomics, Toronto). Tissue sections were deparaffinized by three 5 min washes in xylene, and gradually rehydrated through 2 min washes in 100%, 95%, 80% and 70% ethanol, followed by PBS. Antigen retrieval was performed by
boiling sections for 15 min in 10mM sodium citrate, pH 6.0, in a pressure cooker pre-heated in a microwave oven for 20 min before immersing slides. Blocking and permeabilization was performed by incubation with 0.2% Triton X-100 in PBS containing 3% BSA for 20 min at room temperature.

Sections were incubated with primary antibodies overnight in a humidified chamber at 4°C in 3% BSA in PBS. Antibodies and dilutions are as follows: 1:100 anti-ERα (Santa Cruz; sc-542), 1:100 anti-BrdU (abcam; ab6326), 1:20 anti-Flag (abm; G191) and undiluted anti-Alx4 containing supernatant (produced in our lab). After three washes with PBS, sections were incubated with fluorescently-labelled secondary antibody for 1hr at room temperature in 3% BSA in PBS. Antibodies and dilutions used were 1:200 Alexa Fluor 568 donkey α-mouse IgG (Invitrogen), 1:200 Alexa Fluor 488 goat α-rabbit IgG (Invitrogen) and 1:200 Alexa Fluor 488 donkey α-goat IgG (invitrogen). Coverslips were washed with PBS and mounted on slides with Vectashield Mounting Medium with DAPI (Vector Laboratories). Slides were viewed using a Nikon Ellipse 80i microscope equipped with a QImaging Fast1394 digital camera and compiled using Qcapture Pro 6.0 (QImaging).

Primary Cell Culture

Primary mammary epithelial and mesenchymal cells were isolated as described previously (Chang et al., 2012; Niranjan et al., 1995). Briefly, wild type female mice on the C57Bl/6N background were sacrificed at 3 months of age. Thoracic and inguinal mammary glands were dissected and then minced with a razor blade into small fragments. Next, the glands were incubated in a solution of 3mg/ml collagenase A (Roche) and 75μg/ml trypsin (Sigma-Aldrich) in serum-free DMEM/F12 for 2hr at 37°C. Mechanical dissociation was then performed by slowly pipetting 10-15 times with a 5ml pipette, adding FBS to a final concentration of 2%. Glands were centrifuged at 250rpm for 2min to separate the epithelial (pelleted) and mesenchymal (supernatant) fractions. The mesenchymal layer was then collected, strained using a 40μm cell strainer, washed with DMEM/F12, and plated in DMEM/F12 with containing 10% FBS, 10μg/ml insulin, 10μg/ml transferrin.
and 20ng/ml EGF.

For epithelial cell isolation, the pelleted fraction was resuspended in serum-free DMEM/F12 media containing 1.5mg/ml collagenase A and incubated at 37°C for 30 min. The cells were pelleted by centrifugation, washed in DMEM/F12 and plated in primary culture media mentioned above. Epithelial cells are present as organoids in culture. These large “clumps” of epithelial cells do not attach to plastic culture dishes as quickly as single-cell mesenchymal cells. Pure organoid cultures were obtained by allowing extraneous mesenchymal cells to attach to the plate (~2hr) and then re-plating organoids that were still in suspension or loosely attached.

**Erk1/2 Activation Assays**

For Erk1/2 activation assays, primary mouse mammary epithelial cells were starved in DMEM/F12 media containing 0.2% FBS for 24hr and then stimulated for 15 min with pcDNA3-conditioned media, Shh-conditioned media, or Shh-conditioned media with 20nM SANT-1 (Toronto Research Chemicals), or 10nM PP2 (Sigma-Aldrich). Inhibitors were added to cells 1hr prior to Shh-stimulation. Cells were depleted of E2 in Erk1/2 activation assays that required ERα activation or inhibition. Primary epithelial cells were cultured in phenol red-free Opti-MEM (Corning) containing 10% charcoal striped FBS (cFBS) (Gibco) for 24hr, followed by serum starvation in media containing 0.2% cFBS for 24hr. Cells were then stimulated with N-Shh conditioned media (phenol-red free, cFBS) alone or in the presence of 10nM ICI 182 780. For canonical pathway activation assays, primary mammary epithelial and mesenchymal cells were starved in DMEM/F12 containing 0.2% FBS for 24hr, and 0.5% FBS for 48hr, respectively. Cells were then stimulated with 1µg/ml N-Shh peptide (R & D Systems) or with N-Shh peptide and 20nM SANT-1 for 24hr.

**Preparation of Shh-Conditioned Media**

Shh-conditioned media was prepared as previously described (Capurro et al., 2012; Harvey et al., 2014). Shh- and pcDNA-conditioned media were prepared by transfecting 40% confluent
100 mm plates of HEK 293 cells in 10% FBS with 10ug of pcDNA3.1-N-Shh (gift of J. Filmus, Sunnybrook Health Sciences Centre) or pcDNA3 using 2mg/ml Polyethylenimine (PEI) at a 2:1 ratio. Cells were grown for 24hr before the media was switched to 5% FBS for 72hr. The media was collected, centrifuged at 2500 rpm for 5 min at 4°C and harvested. The supernatant was then sterile-filtered using a 0.22μm syringe filter. Prior to use, conditioned media was diluted in serum free DMEM to a final serum concentration of 0.5% or 0.2%. Activity of conditioned media was measured by luciferase assay in Shh Light II fibroblasts.

**Luciferase Assays**

Activity of the Shh-conditioned media and N-Shh peptide was assayed using Shh Light II fibroblasts. These cells contain an 8X-Gli-binding-site firefly Luciferase reporter transgene and a constitutive renilla luciferase transgene. Confluent cells were serum-starved in 0.5% serum for 24hr and then stimulated with Shh-conditioned media or N-Shh peptide for 24hr. Cells were then lysed in passive lysis buffer (Promega) and luciferase activity was measured by Dual-Luciferase Reporter Assay System (Promega). Gli-reporter activity was normalized to renilla activity.

**Western Blotting**

Adherent primary mouse mammary epithelial cells in 60 mm culture dishes were washed in ice cold PBS. Cells were then scraped following the addition of 50μl of 1% NP40 lysis buffer (50nM Tris-HCl (pH 8.0), 150mN NaCl, 1% NP-40) containing protease and phosphatase inhibitors (0.57mM PMSF, 10μM leupeptin, 0.3μM aprotinin, 10mM NaF, 1mM sodium orthovanadate). Lysates were incubated for 20 min on ice and then centrifuged in a refrigerated microcentrifuge for 30 min at 13,000 rpm to pellet insoluble material. Supernatant protein concentrations were measured by Bradford assay using Bio-Rad Protein Assay reagent. 4x SDS-loading buffer (50mM Tris pH 6.8, 100mM DTT, 2% SDS, 0.1% 36 bromophenol blue, 10% glycerol) was added to lysates containing 40μg of protein and boiled for 5 min. Samples were resolved by 10% SDS-PAGE and blotted onto nitrocellulose membrane. Blots were blocked with 5% skim milk powder in PBS,
and then probed with primary antibody overnight at 4°C. Antibodies and dilutions used were as follows: 1:1000 mouse α-p-Erk1/2 (Cell Signaling) and 1:1000 rabbit α-Erk1/2 (Cell Signaling). Blots were washed 3x in TBS-T (137mM NaCl, 2.7mM KCl, 25mM Tris, 0.1% Triton X-100) for 15 min each and probed with secondary antibody for 1hr at room temperature. Antibodies and dilutions were as follows: 1:5000 horse α-mouse IgG (Cell Signaling) and 1:5000 goat α-rabbit IgG (1:5000). Protein levels were detected using BioRad-ECL substrate and analyzed using the MicroChemi 4.2 chemiluminescence imaging system (DNR Bio-Imaging Systems) equipped with GelCapture Image Software (DNR Bio-Imaging Systems). Blots to be re-probed were stripped in stripping buffer (2% SDS, 62.5mM Tris pH 6.8, 100mM β-mercaptoethanol) for 20 min at 72°C.

qRT-PCR

Total RNA was isolated from primary mouse mammary epithelial and mesenchymal cell using Trizol reagent (Invitrogen). DNase treatment (Fermentas) was performed on 400ng of RNA from each sample. Reverse transcription was then performed using a SuperScript II Reverse Transcriptase (Invitrogen) and random hexamer primers (Fermentas). A 25X dilution of the resulting cDNA was subjected to qPCR using an iQ SYBR Green Supermix (Bio-Rad) with 10μl reactions. All reactions consisted of 40 cycles with 30 seconds denaturation at 72°C and 30 seconds annealing and 30 seconds extension at 60°C. Primer specificity was confirmed by melt curve analysis. Results were quantified using the ΔΔCt method corrected for primer efficiency with Arbp as a reference gene (Harvey et al., 2014). Primers adapted from previously published sequences (Webster et al., 1995; Zhang et al., 2009) were used as follows:

- mouse Arbp forward 5’ GAAAATCTCCAGAGGCACCATTG 3’;
- mouse Arbp reverse 5’ TCCCACCTTTGCTCCAGTCTTTAT 3’.
- mouse Gli1 forward 5’ CCCATAGGGTCTCGGGGTCTCAAAC 3’;
- mouse Gli1 reverse 5’ GGAGGACCTGCGGCTGACTGTAA 3’;
- mouse Ptch1 forward 5’ GGTGGTTTCATCAAAGTGTGAC 3’;
- mouse Ptch1 reverse 5’ GCCATAGGCAAGCATCAGTA 3’
Results

Forced ERα expression rescues the mes mammary phenotype

Our lab has shown previously (Chang et al., 2012) that the blocked mammary gland development in mes mice can be overcome by epithelial-restricted expression of an activated c-src (c-src<sup>Act</sup>) transgene under the control of the MMTV promoter (Webster et al., 1995). This rescue was associated with increased expression of ERα in mammary epithelial cells. To determine if ERα acts genetically downstream of Ptch1, we employed a transgenic mouse model in which we over-expressed ERα in the mammary glands of mes mice.

CERM mice harbour an inducible flag-tagged murine ERα allele under the control of the tet<sup>O</sup>, as well as the rtTA transgene under the control of the MMTV promoter (Hruska et al., 2002). Luminal epithelial cell-restricted expression of the ERα transgene is facilitated by administration of doxycycline. CERM mice were bred onto the mes background to produce mes/CERM compound mice, which were then backcrossed onto the C57Bl/6N background. To determine if forced expression of ERα could overcome the block to ductal elongation in mes mice at puberty, mice were fed doxycycline continuously from puberty and mammary gland development assessed. As the whole mounts in Figure 7A illustrate, mammary glands in mes mice at twenty weeks remained undeveloped. In these glands, the fat pad remained clear of ductal development, with only the rudimentary ductal structure that forms during embryogenesis present. No TEBs were evident (Figure 7A’), indicating that the mammary ducts were not elongating. In contrast, ductal elongation was apparent in doxycycline-fed mes/CERM littermates (Figure 7B). At this stage, ducts had penetrated the fat pad and had extended past the lymph node. The presence of TEBs (Figure 7B’) suggested that these ducts were elongating at this point in time, similar to what is observed in wild type mice during puberty. These results showed that constitutive expression of ERα in mammary epithelial cells appears to rescue the blocked ductal morphogenesis in the mes mammary gland, albeit, with delayed kinetics.
Although forced expression of ERα drove ductal elongation in the mammary glands of mes mice, this development occurred much later than in the mammary glands of wild type mice. To document the progression of ductal elongation in mes/CERM mice, mammary glands were removed from euthanized age-matched doxycycline-fed mes/CERM, mes and wild type mice at varying ages post-puberty and whole mounts prepared (Figure 8). Glands of age-matched mes/CERM mice that were not fed doxycycline were also analyzed as a control to show that the mammary development in these animals was indeed a product of constitutive ERα transgene expression, and not an inherent trait of our mes mice. As we reported previously (Chang et al., 2012), no mammary gland development beyond the primitive ductal structures established during embryogenesis was seen in mes mice, even by thirty weeks (Figure 8E). Mammary glands from
wild type mice displayed complete morphogenesis by ten weeks of age (Figure 8D). For these latter mice, ductal structures filled the entire fat pad and TEBs were absent (Figure 8D, see insert), indicating that these glands had completed the pubescent developmental stage of mammary development. In contrast, at ten weeks, only rudimentary ductal structures resembling those of their mes littermates were seen for doxycycline-fed mes/CERM mice (Figure 8A). No TEBs were present (Figure 8A, see insert). Between fifteen and twenty weeks of age, the mammary ducts in doxycycline-fed mes/CERM mice had begun to penetrate the fat pad to different extents.
(Figure 8B). TEBs were present (Figure 8B, see insert), suggesting that ductal structures were still elongating. In doxycycline-fed mes/CERM mice between twenty four and thirty weeks of age (Figure 8C), the mammary gland ducts had reached the limits of the fat pad and TEBs were no longer present. The mature ductal structure comprised of a dense network of ducts that exhibited secondary and tertiary branching, resembling the ductal structures found in mammary glands of wild type mice. The dependence of mammary gland development on doxycycline-induced ERα was verified in mes/CERM animals where doxycycline was withheld. No ductal morphogenesis was evident in these uninduced animals (Figure 8F). Thus, initiation of ductal elongation was not observed in these animals prior to fifteen weeks of age, and development was not completed until at least twenty-four weeks. These data confirm that forced expression of ERα in mammary luminal epithelial cells rescues mammary development in mes mice, albeit with delayed kinetics.

To determine if the rescue by Tet<sup>0</sup>-ERα expression was significant, age-matched groups (15 weeks to 30 weeks of age) of doxycycline-fed mice, including wild type, wild type/CERM, mes and mes/CERM mice, were compared based on the presence or absence of ductal outgrowth (Table 1). Animals younger than fifteen weeks of age were omitted from comparison due to lack of development of mes/CERM mammary glands. Statistical significance was determined using Fisher’s exact test. Statistical significance between mammary gland development in induced mes/CERM mice compared to mammary gland development of mes littermates was defined by p<0.05. These data show that forced ERα expression overcomes blocked mammary gland development in mes mice where the Ptch1 protein is truncated at its C-terminus. Our data suggests that ERα acts genetically downstream of Ptch1.

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<tr>
<th>Genotype</th>
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<td>0</td>
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</tr>
<tr>
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<td>*p=0.0455</td>
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Table 1. Summary of ERα rescue of mes mice. Mammary gland development in doxycycline-fed wild type, wild type/CERM, mes and mes/CERM mice that were older than fifteen weeks of age. Glands were compared based on the presence or absence of ductal outgrowth and analyzed by Fisher’s exact test. Differences in the presence of ducts were significant between mes and wild type mice, mes and mes/CERM mice.
To determine if the mammary ducts of mes/CERM mice exhibited normal epithelial organization, we performed immunofluorescence staining on sections of mammary glands and probed for the cell type-specific markers, ERα and p63 (Figure 9). While ERα is expressed in both the stromal and epithelial cellular compartments in the mouse mammary gland, it is specific to the luminal epithelial cells and is not expressed in myoepithelial cells (Shyamala et al., 2002). In contrast, p63 is a myoepithelial cell-specific marker (Batistatou et al., 2003). Immunofluorescence was performed on paraffin-embedded sections of mammary glands from adult wild type, wild type/CERM, mes and mes/CERM mice that were fed doxycycline. All animals exhibited a single outer layer of p63 positive cells surrounding the innermost layers of luminal epithelial cells that were negative for p63 expression (Figure 9C, G, K & O). Expression of ERα was restricted to the luminal epithelial cells, evident by the lack of overlap between signals for ERα with p63 (Figure 9D, H, L & P). Furthermore, in expressing cells, ERα was localized to the nucleus in all animals.

In order to identify differences in ERα expression between mammary glands of mes, mes/CERM and wild type mice, we counted ductal epithelial cells that were positive for ERα staining in age-matched adult animals (eighteen to twenty-two weeks of age). Data was analyzed by one-way ANOVA followed by pairwise comparison of means using Tukey’s Honest Significant Difference test. Approximately 40% of luminal epithelial cells were positive for ERα in wild type mice (Figure 9Q). As we demonstrated previously (Chang et al., 2012), reduced ERα expression was also observed in mes mice (Figure 9E-H) compared to wild type littermates (Figure 9A-D). Only 10-20% of epithelial cells in ducts from mes mammary glands stained positively for ERα (Figure 9Q). We observed an approximate 2-fold increase in the percentage of cells with nuclear ERα expression in induced mes/CERM mice relative to mes mice (p<0.05). Furthermore, the expression of TetO-ERα increased ERα expression to levels resembling those of wild type mice (Figure 9Q). In fact, expression levels and subcellular localization of ERα were indistinguishable between wild type (Figure 9A-D) and mes/CERM (Figure 9M-P) littermates. Thus, these data show that expression of ERα transgene restores ERα expression in mes mice to levels observed in wild type littermates. Furthermore, although mammary gland development of mes/CERM mice
was distinct from that of wild type mice, ducts in mammary glands of mes/CERM mice exhibited normal cellular organization and expression patterns for ERα.

**Figure 9.** *mes/CERM ducts demonstrate normal cellular organization and rescued ERα expression.* Immunofluorescence staining for ERα and p63 in 20 week old virgin wild type (A-D), *mes* (E-H), wild type/CERM (I-L) and *mes/CERM* (M-P) mice. No co-localization of ERα is observed with p63 (D, H, L & P). Reduced ERα expression is apparent in *mes* mice (F) compared to wild type (B). ERα expression is increased, however, in *mes/CERM* mice (N), but expression levels do not differ significantly between *mes/CERM* and wild type littermates. Q) ERα-positive cell percentages were determined by cell counting; 10 fields per animal, n=3 for all animal groups. Data was analyzed by one-way ANOVA followed by comparisons of means using Tukey’s multiple comparison’s test. *p<0.05. Scale bar: 25μm.
Forced ERα expression induces a proliferative state in the mammary gland

We performed immunofluorescence staining on sections of mammary glands taken from induced and uninduced adult wild type/CERM mice to detect ERα transgene expression (Figure 10A). Paraffin-embedded section of mammary glands taken at twenty weeks of age were probed with antibodies against Flag to detect transgene expression, as well as with antibodies against ERα (Figure 10A). Flag staining was observed in small populations of epithelial cells in ducts from induced wild type/CERM mice. In contrast, no signal for Flag was detected in ducts from

![Image](image_url)

**Figure 10.** Tet-O-ERα drives cellular proliferation in the mammary gland in a cell non-autonomous fashion. Immunofluorescence staining for Flag and ERα (A) and Flag and BrdU (B) in mammary glands of induced and uninduced adult wild type/CERM mice. ERα transgene expression was detected with antibodies against Flag in doxycycline-fed mice (A & B). Cells positive for Flag staining were also positive for ERα (A). BrdU incorporation was observed in doxycycline-fed mice (B) although no Flag staining was detected in BrdU-incorporating cells.
wild type/CERM mice that were not fed doxycycline. Cells expressing detectable levels of Flag-
ERα also stained with an anti-ERα antibody (Figure 10A). The levels of ERα detected by the
anti-ERα antibody, however, were heterogeneous. Both weak and strong staining for ERα was
observed in cells that also stained positively for Flag. These results show that Flag-ERα expression
is dependent on doxycycline administration, and that this induction results in heterogeneous ERα
protein expression.

Given that animals expressing the ERα transgene exhibited deregulated ERα expression
(Figure 8; (Frech et al., 2005)), as well as irregular ductal elongation occurring well after the
end of puberty (Figure 7), we sought to determine if transgene expression caused aberrant cell
cycle progression in adult mammary glands (Figure 10B). To determine the proliferative-status of
mammary ducts, we injected adult induced and uninduced wild type/CERM mice with 50μg/kg
BrdU 2hrs prior to being euthanized. We then probed sections of mammary glands with antibodies
against Flag and BrdU. As expected, Flag staining was only observed in glands from animals that
had received doxycycline (Figure 10B). A fraction of cells in every duct showed BrdU incorporation
in induced wild type/CERM mammary glands. In contrast, no BrdU incorporation was observed
in animals that had not received doxycycline treatment. Interestingly, cells expressing detectable
levels of Flag were not associated with cells that incorporated BrdU in the induced wild type/
CERM mammary glands. Specifically, cells that were positive for Flag were distinct from cells
that incorporated BrdU. These results suggest that the ERα transgene drives proliferation in the
mammary gland in a cell non-autonomous fashion.

We performed immunofluorescence staining to further determine if ERα transgene
expression drives cell cycle progression on the mes background (Figure 11). We probed mammary
gland sections with antibodies against BrdU and ERα from twenty week old doxycycline-fed wild
type (Figure 11A-D), wild type/CERM (Figure 11E-H) and mes/CERM mice (Figure 11M-P).
Age-matched wild type/CERM mice not fed doxycycline were also analyzed as a control for any
doxycycline-independent effects of ERα transgene (Figure 11I-L). BrdU incorporation was absent
in the mammary glands from adult wild type mice (Figure 11A-D). In contrast, a fraction of cells
in every duct in doxycycline-fed wild type/CERM (Figure 11E-H) and mes/CERM (Figure 11M-P) mice stained positively for BrdU, indicating the presence of cycling cells. The lack of BrdU incorporation in adult wild type/CERM mice not fed doxycycline (Figure 11I-L) indicated that the presence of cycling cells in induced mes/CERM and wild type/CERM mice was the result of MMTV-driven ERα transgene expression. No cells in mammary glands from doxycycline-fed wild type animals stained positively for BrdU (Figure 11A-D) confirming that doxycycline administration alone (or itself) did not induce proliferation of mammary epithelial cells. Thus, constitutive expression of the ERα transgene in both wild type and mes adult mice drives cell cycle progression in mammary epithelial cells. Although we previously showed that cells that had
incorporated BrdU did not express Flag-ERα (Figure 10B), we detected heterogenous ERα staining in cells that also showed BrdU incorporation (Figure 11). These data suggested that cells expressing ERα are cycling, and that this proliferation is stimulated by induction of the ERα transgene. These results suggest further that ERα transgene generated an overall proliferative state in the mammary gland, rather than acting in a cell autonomous fashion to drive specific cells through the cell cycle.

To further investigate the overall proliferative state of the mammary gland, changes in the expression of the stromally-restricted, homeodomain protein, Alx4, were assessed. Alx4 is induced in stromal fibroblasts during puberty and pregnancy but is not expressed in the resting adult mammary gland (Hudson et al., 1998; Joshi et al., 2006). Administration of E2 to pre-pubescent female mice induces Alx4 expression in these fibroblasts. Furthermore, the absence of Alx4 expression in stromal fibroblasts that occurs the mutant mouse strain, Strong’s luxoid, causes cell non-autonomous defects in mammary ductal morphogenesis during puberty. Thus, expression of Alx4 in stromal cells of CERM or MMTV-c-src\textsuperscript{Act} mice on both wild type and mes backgrounds was determined by immunofluorescence. As Figure 12A-B illustrates, Alx4 expression was absent

![Figure 12. ERα and c-src\textsuperscript{Act} induced expression of Alx4 in the mammary stroma of adult wild type, but not mes, mice. Mammary glands of wild type (A-B), wild type/c-src\textsuperscript{Act} (C-D), wild type/CERM (E-F), mes (G-H), mes/c-src\textsuperscript{Act} (I-J), mes/CERM (induced) (K-L) virgin twenty-four week-old adult female mice were stained for Alx4. Staining is absent in wild type mammary ducts. Expression of constitutive c-src\textsuperscript{Act} or conditional ERα in ductal epithelial cells induces stromal expression of Alx4. Scale bar: 25μm]
in the adult wild type stromal cells adjacent the epithelial ducts. However, in wild type mice with induced ERα (Figure 12E-F) or constitutive c-src\textsuperscript{Act} expression (Figure 12C-D) in mammary epithelial cells, an induction of Alx4 expression in adjacent stromal fibroblasts was apparent. In contrast to Tet\textsuperscript{O} - ERα or MMTV-c-src\textsuperscript{Act} on the wild type background, on the mes background, Alx4 expression could not be detected despite expression of either ERα or c-src\textsuperscript{Act} (Figure 12I-J and K-L, respectively). Thus, the luminal epithelial cell-restricted induction of either ERα or c-src\textsuperscript{Act} appeared to generate a signal, emanating from mammary epithelial cells, that stimulated stromal fibroblasts to express Alx4. The induction of Alx4 expression in these fibroblasts was dependent, however, on the presence of wild type Ptch1.

**Hh signalling in primary mammary epithelial cells and fibroblasts**

Several groups have published data pertaining to the effects of deregulated Hh-pathway activation on the development and transformation of the mammary gland (for review (Cui et al., 2010; Hui et al., 2013; O’Toole et al., 2009)). Although the cell types involved in Hh-signalling in the mammary gland have been identified (García-Zaragoza et al., 2012), to our knowledge, comparison of activation through the canonical, Smo-dependent pathways has not been performed for primary mammary epithelial versus mesenchymal cells. We performed activation assays in isolated mammary epithelial and mesenchymal cells from three month-old wild type mice to determine the ability of these cells to respond to N-Shh through the canonical signalling system (Figure 13). Following serum starvation, we stimulated cultured primary cells with 1ug/ml N-Shh peptide, heat killed N-Shh peptide, or N-Shh peptide with 20nM SANT-1 for twenty-four hours. We then performed qRT-PCR to quantify expression levels of two Hh-pathway target genes, *Ptch1* and *Gli1*. We observed a 7-fold and 25-fold increase in *Ptch1* and *Gli1* mRNA levels, respectively, in primary mammary mesenchymal cells following twenty-four hours of N-Shh stimulation (Figure 13A). With both targets, activation of mRNA expression was completely inhibited in cells treated with N-Shh in the presence of SANT-1. These results confirm that Smo-dependent activation of the canonical Hh-pathway occurs in the mesenchymal cells of mammary glands in wild type mice.
We observed a different response to N-Shh stimulation in primary mammary epithelial cells (Figure 13B). Unlike in the mesenchymal cells, a very limited or no induction of \textit{Gli1} and \textit{Ptch1}, respectively were observed for primary epithelial cells. Specifically, a small but significant 3-fold increase in \textit{Gli1} levels was observed after twenty-four hours, while no induction of \textit{Ptch1} was observed. Treatment with SANT-1 repressed the N-Shh-mediated \textit{Gli1} induction. These results reveal a differential ability of specific cell types in the mammary gland to respond to canonical Hh signalling. While the primary mesenchymal cells demonstrated a robust canonical response to N-Shh stimulation, the mammary epithelial cells are relatively refractory to stimulation through this pathway.

The limited ability of primary mammary epithelial cells to respond to canonical Hh signalling is consistent with what we have observed previously in human breast and breast cancer cell lines (Chang, unpublished data). One of these cell lines, MCF10A, which is refractory to
canonical Hh signalling, lacks detectable Smo expression (Chang et al., 2010; Zhang et al., 2009). We have also shown that N-Shh can elicit activation of signalling factors, such as c-src and Erk1/2 through a pathway that is independent of Smo activity in these cells (Chang et al., 2012, 2010; Harvey et al., 2014). These results suggest that these cells signal through a Smo-independent, non-canonical system in response to Shh that activates c-src and Erk1/2. We next sought to determine if primary mammary epithelial cells signal through these non-canonical systems. To determine if non-canonical activation of the MAPK pathway occurs in mammary epithelial cells, we stimulated

**Figure 14. N-Shh activates Erk1/2 independently of Smo in primary mouse mammary epithelial cells.** Primary mammary epithelial cells isolated from wild type mice at three months of age were serum starved for 24h and then treated with Shh-conditioned media, pcDNA3-control media, or Shh-conditioned media with ICI 182 780, Sant-1 or PP2 for 1h. Blots were probed for activated (phospho)-Erk1/2 and then reprobed for total Erk1/2. A) Activation of Erk1/2 was observed 15 min following stimulation by sonic hedgehog. C) Addition of ICI 182 780 1hr prior to Shh stimulation partially inhibits Erk1/2 activation. E) Cells were treated with 20nM SANT-1 or 10nM PP2 for 1 hr before Shh stimulation. PP2, but not Sant-1 treatment inhibits Shh-dependent Erk1/2 activation. Quantification of Erk1/2 activation are shown (B, D & F). Data were analyzed by two-way ANOVA followed by pairwise comparison of means using Dunnett’s multiple comparisons test. Data are displayed as mean ± SEM, n= 3 for all groups, *p < 0.05.
isolated primary mouse mammary epithelial cells with N-Shh conditioned media (Figure 14). As Figure 14A illustrates, a 4-fold increase in the levels of Erk1/2 phosphorylation was observed fifteen minutes following stimulation with Shh compared to baseline phosphorylation levels of unstimulated cells (p<0.05). This phosphorylation was reduced by thirty minutes (Figure 14B). This activation was partially inhibited when cells were stimulated with Shh in the presence of the ERα SERD, ICI 182 780. As Figure 14C illustrates, a 4.5-fold increase in activated Erk1/2 was observed in cells treated with Shh in comparison to serum starved cells alone. However, only an approximately 2-fold increase in activated Erk1/2 was observed in cells stimulated with N-Shh in the presence of ICI 182 780 (Figure 14D). The inhibitory effects of ICI 182 780 on N-Shh treated Erk1/2 activation was statistically significant (p<0.05). These results show that Erk1/2 activation by N-Shh occurs in a signalling pathway that is partially dependent on ERα. Shh-mediated activation of Erk1/2 was completely abolished when cells were treated with the c-src inhibitor, PP2, but not the Smo inhibitor, SANT-1 (Figure 14E). In contrast to cells treated with SANT-1, which demonstrated Erk1/2 activation comparable to cells treated with Shh alone, phosphorylation of Erk1/2 remained at control levels in cells treated with both PP2 and Shh (Figure 14F). This inhibition was statistically significant (p<0.05). Thus, these results show that while relatively refractory to canonical Hh signalling, primary mammary epithelial cells show activation of a Hh signalling pathway that activates Erk1/2 and that this non-canonical pathway requires the activities of ERα and c-src, but not Smo.
Discussion

*Forced ERα expression rescues the blocked mammary gland development in mes mice*

The Hedgehog (Hh)-signal transduction pathway is a fundamental regulator of tissue development and homeostasis. This signalling system is regulated by the ligand receptor, Ptch1, which upon Hh-binding, activates signal transduction through the reversal of inhibition of Smo-activity and subsequent activation of Gli-family transcription factors. Genetic studies have suggested that Hh-signalling is either absent or repressed in the mammary gland during development (Hatsell and Cowin, 2006). This state is in contrast to the development of other epidermal appendages, such as the hair follicle and teeth, in which the requirement for Hh signalling is well established (Cho et al., 2011; Michno et al., 2003). We show here that Hh-signalling, albeit through a pathway, at least partially independent of the ‘canonical’ pathway, is absolutely required for pubescent mammary gland development.

Upon careful examination of the sequence of murine Ptch1, our lab previously showed that the C-terminal region of Ptch1 contains highly conserved sequences that encode consensus binding sites for Class I and Class II SH3-domains, as well as WW-domains (Chang et al., 2010). We further showed that a number of factors harbouring these domains can associate with the cytoplasmic C-terminal region of Ptch1, including the SH3-domains of c-src, Grb2 and PIK3R2 (p85α), as well as WW domain-harbouring E3 ubiquitin ligases, WWP2 and Smurf2. These results are supported by the recent finding that *Drosophila* Smurf (dSmurf) complexes with the C-terminus of *Drosophila* Ptch (dPtch1) through motifs in dPtch that are present in the vertebrate homologues (Huang et al., 2013).

The *mesenchymal dysplasia (mes)* mouse has been useful for investigation of the requirement of the Ptch1 C-terminus in Shh signalling pathways. These mice harbour a 32-bp deletion in the second to last exon of Ptch1 (Sweet et al., 1996). This protein has deleted the last 220 amino acids in its C-terminus replacing it with a small, random sequence of 68 amino acids (Makino et al., 2001; Sweet et al., 1996). Homozygous mes mice exhibit blocked mammary gland development, revealing a mammary-gland specific role for Hh-signalling (Moraes et al., 2009). Despite this
drastic phenotype, no changes in the levels of Ptch1 are detected in the epithelial cells or fibroblasts isolated from mes mammary glands, suggesting that canonical signalling is not significantly altered in this tissue. Furthermore, the Ptch1mes protein binds Shh-ligand with an affinity similar to wild type Ptch1 (Nieuwenhuis et al., 2007) and appears to facilitate signalling through the canonical Hh-signalling pathway similar to cells with the full length Ptch1 protein (Harvey et al., 2014). Thus, the basis for the defects in mammary gland development in mes mice is not apparent.

For some tissues, mes protein appears to act as a hypomorphic variant of Ptch1, as activated Hh-signalling, determined by increased expression of Hh-target genes is observed (Li et al., 2008). In contrast, similar to what we observed for the mammary gland, no apparent impairment of the inhibition of Smo-activity was observed in the dermis of mes mice, despite altered morphogenesis of the hair follicles (Nieuwenhuis et al., 2006). In contrast, increased mGli1 and mPtch1 expression occurs in epididymal white adipose tissue (Li et al., 2008). Polydactyly exhibited by mes mice is also consistent with altered activity of the canonical Hh-signalling pathway (Masuya et al., 1997).

We showed previously that the defect in the mes mammary gland can be rescued, albeit with delayed kinetics, by the constitutive expression of activated c-src (c-src^Act) (Chang et al., 2012). A significant increase in the levels of ERα expression was observed in mammary epithelial cells of the mes/MMTV-c-src^Act mice relative to mes mice alone. These data suggest that Hh-signalling in the mammary gland may require, at least in part, signalling through pathways that control the expression and/or the activities of ERα. If ERα is acting downstream of Hh in this pathway, forced expression of ERα in mes mice should also rescue the mes mammary phenotype. As we demonstrate here, conditional expression of an ERα transgene under the control of the MMTV promoter also overcomes the block to mammary morphogenesis with kinetics similar to that of the MMTV-c-src^Act allele. These results suggest that ERα and c-src operate in a signalling cascade downstream of Ptch1, and that this signalling system is impaired by the truncation of Ptch1 in mes mice.

We previously demonstrated that the block to mammary morphogenesis in mes mice was not due to a lack of ovarian hormone production. This was supported by the normal appearance of
ovaries in mes mice (Chang et al., 2012), as well as the ability of a small number of mes females to become pregnant and give birth to small litters (Sweet et al., 1996). Instead, we suggested that the mes mammary phenotype was the result of an inability of mammary epithelial cells to respond to ovarian hormones. This was supported by the lack of development in ovariectomized mes mice that were injected with exogenous E2 (unpublished observation). Furthermore, we showed that mes mammary epithelial cells express less ERα than wild type cells (Chang et al., 2012). This reduced expression may be the underlying cause of the lack of response to E2 in mes mammary glands, as the epithelial pool of ERα, as determined through transplantation experiments, is required to drive mammary morphogenesis during puberty (Mallepell et al., 2006).

The rescue of mes mammary gland development by epithelial-restricted c-src^Act was accompanied by an increase of epithelial ERα expression, which further suggested that ERα levels are important for driving mammary morphogenesis and that a signalling cascade, which includes c-src, is involved in maintaining these levels. The truncation of Ptc1 in mes mice, however, abrogates this signalling cascade. Our data here further support this hypothesis. Here, we counted ERα positive luminal epithelial cells and determined that the ducts of mes mice had approximately 2-fold fewer ERα positive cells compared to wild type ducts. The number of ERα-positive cells increased by approximately 2-fold in mes mice expressing the ERα transgene, returning the number of ERα expressing cells to that observed in wild type controls. These results suggest that transgene expression may have increased the ERα expression in mes mammary glands to a level that can support E2 signalling, and therefore, drive ductal elongation. However, although we have verified transgene expression, it is still unclear as to what extent the ERα transgene increases ERα expression in the mammary gland. By knowing the amount of protein being expressed, we will be able to distinguish if the mes phenotype, as well as the rescue by ERα, is a function of the amount of ERα expressed, the number of cells expressing ERα, or both. This could be accomplished through immunoprecipitation, or immunoblotting of isolated primary epithelial cells, as we have already described (Chang et al., 2012).

We performed immunofluorescence to determine that the ductal structures in mes/CERM
mammary glands displayed normal cellular organization. In the mes/CERM mice, we observed proper localization of ERα in the luminal epithelial cells and p63 in the myoepithelial cells. Although the mammary glands of adult mes/CERM mice show structural organization reminiscent of wild type glands, it is unknown whether these glands can support alveolar differentiation or if they are capable of lactation. During pregnancy, progesterone (Pg) is required to induce extensive side-branching and alveologenesis (Brisken et al., 1998). In combination with prolactin (PRL), Pg also promotes the differentiation of the alveoli (Humphreys et al., 1997; Ormandy et al., 1997) which are the structures that synthesize and secrete milk during lactation. Mammary development during pregnancy and lactation has not been investigated in mes mice, as they are largely unable to become pregnant. Furthermore, in addition to ERα, reduced PR expression in the epithelial cells in mes mammary glands has been reported (Moraes et al., 2009). The development of alveoli and subsequent lactation in mes and mes/CERM mice could potentially be investigated through transplantation experiments in which the glands from mes and mes/CERM mice are grafted onto the wild type mice that are impregnated. In this environment, mes and mes/CERM mammary glands will be exposed to endogenous levels of hormones during and after pregnancy.

ERα expression drives proliferation in the adult mammary gland

We demonstrated ERα transgene expression by immunofluorescence. While Hruska et al. have demonstrated the presence of Flag-tagged ERα by immunoprecipitation/western analysis using whole cell lysates derived for the entire the mammary gland (Hruska et al., 2002), to our knowledge, we are the first to identify individual Flag-tagged, ERα transgene-expressing cells in this tissue. We identified a population of epithelial cells that stained positively for Flag in mammary ducts from doxycycline-fed wild type/CERM mice. In contrast, a signal for the Flag epitope was absent in mammary glands from wild type/CERM mice in which doxycycline administration was withheld. There is a disconnect between the number of cells expressing Flag and those expressing ERα. Although we did observe a 2-fold increase in the number of cells expressing ERα in wild type/CERM mice, only a small fraction of these cells also stained positively for Flag. Therefore,
transgene expression may be too low for Flag-detection in these ERα positive cells. One could perform in situ hybridization on mammary sections to determine what percentage of cells are expressing the transgene, at least at the genomic level, and compare these numbers to how many cells are proliferating or how many we can detect with Flag staining.

To determine if ERα transgene expression was driving cell proliferation, we probed mammary gland sections for BrdU. We demonstrated that a percentage of cells in every duct in mammary glands from animals expressing the ERα transgene were positive for BrdU incorporation. Interestingly, no overlapping of signals for Flag or BrdU was observed, suggesting that ERα transgene expressing cells are not proliferating. Several studies have shown that proliferating cells do not express ERα. One possibility for this is that upon activation, E2 stimulates proliferation, but is then rapidly eliminated from cells when they enter the cell cycle. Indeed, several studies have shown rapid loss of ERα from cells in response to E2 (Reid et al. 2003; Cheng et al., 2004). Furthermore, ERα phosphorylation events, mediated by ligand-bound ERα crosstalk with signalling kinases, may facilitate both the receptor’s transcriptional activation to its proteolytic degradation. Recent work showed that c-src accelerates E2-dependent ERα proteolysis (Chu et al., 2007). E2 stimulates rapid c-src activation, and c-src phosphorylates ERα at Y537 to facilitate its binding to E6-AP (Sun et al., 2012), a member of the HECT domain E3 ubiquitin ligase family that also functions as a ligand-activated co-activator for ERα and PR (Ramamoorthy et al., 2010). This complex is then recruited to a subset of ER target gene promoters, leading to their transcriptional activation (Sun et al., 2012). However, since the ERα transgene is not under the same regulatory programs as endogenous ERα, this protein may not be degraded upon activation. This is supported by previous results that ERα transgene expression is not reduced following E2 administration in the mammary gland of CERM mice (Frech et al., 2005).

It is also suggested that cells expressing ERα do not proliferate because activated ERα stimulates cell proliferation in a cell non-autonomous fashion. Transplantation experiments completed by Mallepell et al. demonstrated that reconstitution of mammary glands occur by ER-positive epithelial cells driving proliferation of ERα epithelial cells (Mallepell et al., 2006). These
results suggest a mechanism in which ERα transgene drives proliferation in CERM mice. By rescuing ERα expression in the epithelial compartment, these cells can now support epithelial-mesenchymal cross-talk to stimulate proliferation of adjacent epithelial cells.

**Epithelial-ERα stimulates stromal activation**

We demonstrated both ERα and c-src^Act^ give rise to a Ptch1-dependent activation of the mammary stromal cells in vivo. Specifically, Alx4-expression was probed in mammary glands of wild type mice expressing mammary epithelial-restricted ERα transgene or c-src^Act^ at twenty-four weeks. In these adult mice, the stromal cell-restricted, paired-like homeodomain transcription factor, Alx4, was induced in mammary fibroblasts, whereas the no Alx4 was observed in age-matched wild type littermates. We conclude that ERα and c-src expression in the luminal epithelial cells induced the expression of factors that stimulated the surrounding stromal cells. Interestingly, despite the rescue by ERα and c-src^Act^ in the mes mice, induction of Alx4 expression was not observed in stromal cells of mes mice. Thus, wild type Ptch1 was required for stromal induction of Alx4 by adjacent mammary epithelial cells. The basis for the difference between mes and wild type mice has not been defined. We propose the possibility that ERα and c-src induce the expression of a Hh ligand in the mammary epithelial cells, that stimulates adjacent mesenchymal cells to express Alx4. However, production of this mitogen is dependent on the activities of wild type Ptch1 in these cells. This model can be tested in reciprocal transplantation experiments between mes and wild type mammary glands. Specifically, mes and wild type mammary glands can be graphed onto wild type hosts during puberty. If the C-terminus of Ptch1 is required for stromal Alx4 induction, then we should observe Alx4 expression in the stroma in mice harbouring wild type epithelium. No induction should be observed in the mice harbouring the mes epithelium. Regardless, these data offer further evidence for the role of components of the Hh-pathway in mediating signalling interactions between stromal and epithelial compartments in the developing mammary gland.
Canonical Hh signalling in primary mammary epithelial cells and fibroblasts

We have shown that there is a lack of signalling through the ‘canonical’ pathway in response to the Hh-ligands in human cell lines derived from untransformed breast cells or breast cancer cells, including MCF-7, MDA-MB-231 and MCF10A (Chang et al., 2010 and unpublished observations). To determine if the primary mammary cells are also refractory to canonical Hh signalling similar to human breast cell lines, we stimulated with N-Shh ligand isolated primary epithelial and mesenchymal cells. We observed robust induction of Ptch1 and Gli1 mRNA expression in primary mammary fibroblasts. This expression was sensitive to treatment with SANT-1, demonstrating the requirement of Smo-activity for this signalling. In contrast, the response of primary epithelial cells to N-Shh-ligand was distinct. Under the conditions in primary cell culture, we observed that primary mammary epithelial did not induce expression of the Hh-target gene, mPtch1, and only weakly induced mGli1. Therefore, we show differential Hh signal transduction in two specific mammary gland cellular compartments.

Our findings that primary mammary epithelial cells exhibit restricted canonical Hh signalling in response to N-Shh is at odds from what has been observed in several studies describing the consequences of altered expression of the downstream components of the Hh-pathway (for review, see Che et al., 2013; Hui et al., 2010). Our experiments, however, differ in that these other groups have not examined canonical Hh signalling specifically in differentiated primary mammary epithelial or mesenchymal cells through stimulation by Hh ligands.

O’Toole et al. published a report in which they provide a detailed description of the localization of key Hh pathway components in a large, well-characterized breast cancer cohort by immunohistochemistry (O’Toole et al., 2011). This group suggested a paracrine mode of canonical Hh signalling in breast cancer with high Hh ligand and Gli expression in the epithelial and stromal compartments, respectively. The importance of this paracrine Hh signature is supported by evidence that Hh target gene expression is limited to the stromal compartment in mouse models, and that Hh ligand expression produces no detectable cell-autonomous effect in mammary carcinoma cells in vitro.
The role of Hh signalling was also investigated in the lactating mammary gland by overexpressing Shh in the luminal epithelia (García-Zaragoza et al., 2012). Again, Shh secretory mammary epithelium-derived protein was not found to activate Hh signalling in the Shh producing cells in an autocrine manner. Instead, Hh responsive cells were located adjacent to these secretory epithelial cells as well as in the stroma. Therefore, in the conditions established in this mouse model, Shh signalling was mostly intraepithelial and paracrine. Paracrine Hh signaling, in which Hh secreted by the epithelial cells activates the Hh pathway in adjacent mesenchymal cells, has also been described during the development of the digestive tract and prostate (Mao et al., 2010; Yu et al., 2009) as well as in ovarian (Yauch et al., 2008), prostate (Shaw et al., 2009) and pancreas (Bailey et al., 2009) cancer progression. Intraepithelial Hh signalling has also been described and might play a role in stimulating proliferation of progenitor cells (Beachy et al., 2004; Watkins et al., 2003).

The above mentioned studies may explain the differences in Hh pathway activation in our isolated primary mammary epithelial and mesenchymal cells. In these studies, the mesenchymal cells readily respond to Shh stimulus produced by the adjacent compartment. However, it appears that Shh-producing cells do not exhibit Hh pathway activation. The slight induction of Gli1 mRNA in our primary mammary epithelial cells may be a result of intraepithelial Hh signalling with only a subset of epithelial cells responding to Hh. As Shh producing cells do not respond to Shh stimulus, fewer cells may be available and capable of Hh signalling. We could perform immunofluorescence to determine if the lack of response to Hh stimulus is a function of the response of only a fraction of epithelial cells to Shh. Isolated mesenchymal and epithelial cells could be stimulated with N-Shh, fixed and stained for Ptch1 and Gli1. If the sheer number of cells capable of Hh signalling is the underlying cause for the difference in response to N-Shh between epithelial and mesenchymal cells, one would expect relatively fewer epithelial cells to stain positively for Ptch1 or Gli1 compared to mesenchymal cells.

However, there is the possibility that the modest Hh signalling observed in our primary epithelial cells may be the result of Hh activating signalling in primary mammary stem cells/
progenitors. Other groups have identified a role for Hh signalling in mammary stem cell/progenitor renewal. Specifically, mammosphere assays have determined that activation of Hh signalling using Hh ligand or Gli1/Gli2 overexpression increases mammosphere formation and mammosphere size, which is inhibited by cyclopamine (Liu et al., 2006). Furthermore, mammosphere-forming efficiency of primary mammary epithelial cells is increased in cells harbouring activated Smo under the control of MMTV (Moraes et al., 2007).

Non-Canonical Hh signalling in primary mammary epithelial cells

The lack of canonical Hh signalling observed in primary mouse mammary epithelial cells was consistent with our previous report using untransformed human breast and breast cancer cell lines (unpublished data). Here, Shh can stimulate the activation of the MAPK and c-src pathways. Given that these cells lack detectable Smo expression (Chang et al., 2010; Mukherjee et al., 2006; Zhang et al., 2009), we suggested that this activation occurred through a non-canonical Hh-signalling pathway that operated independently of Smo-activity.

In this thesis, we showed that stimulation by N-Shh of primary mammary epithelial cells activated Erk1/2 in both an ERα and c-src-dependent manner. Inhibition of activation of either factor, but not inhibition of Smo, reduced or blocked the ability of N-Shh to stimulate Erk1/2. Thus, the non-canonical Hh-signalling pathway we identified previously (Chang et al., 2012) appears to involve the activities of ERα and c-src in mammary epithelial cells. The precise mechanism of this activation remains undefined. It is clear, however, that the activities of both c-src and ERα are required for the N-Shh-dependent activation of Erk1/2. In the presence of inhibitors that blocked the activities of ERα or c-src, respectively, N-Shh-dependent Erk1/2 activation was attenuated or blocked completely. The residual activation of Erk1/2 in the presence of ICI 182 780 suggests that while ERα is required for Erk1/2 activation by Shh, other unknown factors independent of ERα may also be involved in Erk1/2 activation. This is not the case with c-src, as we observe total inhibition of Erk1/2 activation in the presence of the c-src inhibitor, PP2. Although other factors may be involved in this signalling system, c-src is absolutely required for the stimulatory effects
of Shh on Erk1/2. No compensation by independent factors was observed. In contrast, Smo is not required for Shh-mediated Erk1/2 activation, as no statistically significant reduction in Erk1/2 activation was observed in the presence of SANT-1.

Rapid, c-src-dependent activation of Erk1/2 by E2 has been demonstrated previously in a number of cell lines. The requirement of other signalling factors was demonstrated by immunoprecipitation and immunoblot studies in the presence of E2 and specific inhibitors. Factors involved in Erk1/2 activation include: Shc (Song et al., 2002), Ras (Migliaccio et al., 1996), c-fos (Di Domenico et al., 1996). Further elucidation of the novel Shh-stimulated activation of Erk1/2 pathway identified here is required, and we can investigate whether these above mentioned signalling factors are involved. Therefore, these data support the existence of a pathway stimulated by the Hh-ligands in mammary epithelial cells that operates through both c-src and ERα, resulting in the activation of the Erk1/2.

**Conclusion**

In summary, this work demonstrates a novel signalling network involving Hh-ligands, c-src and ERα in the regulation of the pubescent mammary gland development. For both MMTV-c-src$^{Act}$ and in CERM mice on the mes background, a significant increase in the levels of ERα relative to mes mice alone are observed in the mammary epithelial cells. The altered expression of ERα suggested that the level of its expression required for ductal morphogenesis at puberty in response to 17β-estradiol requires the normal activity of the Ptch1 C-terminus. This model does not preclude the possibility that the canonical Hh-pathway, signalling through Smo, is also required. This possibility has not been formally tested in the mouse mammary gland. However, mice harbouring conditional c-src$^{Act}$ or ERα alleles are sufficient to overcome the block to the pubescent mammary morphogenesis and is associated with increased levels of ERα relative to mes mice alone.

To conclude, combined with our data characterizing the effect of the MMTV-c-src$^{Act}$ allele on development in the mes mammary gland, our data suggest that a novel Hh-signalling pathway
involving the activities of ERα and c-src are required for the mammary gland morphogenesis at puberty. These data suggest further that, for mammary epithelial cells, non-canonical pathways rather than the canonical Hh-pathway operating through Smo are required for branching morphogenesis at this stage of mammary gland development.

**Figure 15. Novel Hh signalling cascade involving ERα and c-src in pubescent mammary gland development.** Constitutive expression of c-srcAct and ERα under the MMTV promoter rescues blocked mammary gland development in mes mice during puberty. ERα and c-src act genetically downstream of Ptch1 in a signalling cascade downstream of Ptch1 in the mammary gland.
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