THE ROLE OF NHERF1/EBP50 IN WNT SIGNALING IN THE EPITHELIUM

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The Na+/H+ Exchanger Regulatory Factor 1 (NHERF1) is a PDZ-domain containing scaffold abundantly expressed in epithelial tissues. Previous reports from our laboratory demonstrate that NHERF1 negatively regulates Wnt canonical signaling in breast epithelial cells through its interactions with Frizzled receptors. Parallel studies conducted at that time also suggested that NHERF1 knockout mice presented a form of communicating hydrocephalus reminiscent of phenotypes associated with mutations in the non-canonical Wnt Planar Cell Polarity (Wnt PCP) pathway. This observation leads to the hypothesis that NHERF1 regulates both canonical and non-canonical Wnt signaling and acts as a "signaling switch" between the two pathways. To further elucidate the role of NHERF1 in Wnt signaling regulation we proposed three specific aims: 1) To demonstrate that NHERF1 can act as a tumor suppressor in a Wnt/β-catenin driven breast cancer model, 2) To determine the role of NHERF1 expression and Wnt/β-catenin in endocrine resistant breast cancer, and 3) Investigate the role of NHERF1 in the regulation of Wnt PCP signaling in the mouse ependyma. Although the exact role that NHERF1 plays in the regulation of Wnt canonical signaling in breast cancer remains elusive, our results indicate that NHERF1 increases the association of key Wnt PCP members Van-Gogh like (Vangl) and Frizzled (Fzd) and promotes PCP signaling and ciliogenesis in mouse ependymal tissue.

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1.0 INTRODUCTION

This thesis aimed to further characterize the role of the PDZ scaffold NHERF1 in the regulation of Wnt signaling in epithelial tissues; specifically the mammary and ependymal tissues. This chapter will introduce several of the proteins, signaling pathways and pathologies relevant to the research discussed in chapters 2 and 3.

1.1 INTRODUCTION TO PDZ PROTEINS

PDZ domains are abundant protein-protein interaction domains named after the first three proteins in which they were discovered: **P**SD-95, **D**iscs-large and **Z**O-1 [1]. In humans, there are approximately 200 proteins that contain PDZ domains and nearly a third of these proteins contain more than one. PDZ domains are typically 80-90 amino acids long and they fold into compact globular structures composed of six β-sheets (βA-βF) and two α-helixes, one short (αA) and one long (αB) [2]. Most PDZ ligands are four amino-acid sequences and are located at the extreme C-terminus of the target protein; however, internal PDZ ligands upstream of the C-

terminus have also been described [3]. Ligand residues are typically labeled by their position in relation to the C-terminus, where the C-terminal residue is referred to as P^0, while subsequent residues towards the N-terminus are named P^{-1}, P^{-2}, P^{-3} etc. PDZ domains are classified by the type of amino acid sequences they typically bind to: Class I PDZ domains interact with proteins ending in the consensus sequence $-X^{-3}$-S/T^{-2}-X^{-1}-ϕ^0, where X is any amino acid and ϕ is a hydrophobic residue. Class II domain bind to X^{-3}-ϕ^{-2}-X^{-1}-ϕ^0 sequences and class III domains prefer to bind to D/E/K/R^{-2}-X^{-1}- ϕ^0 motifs [4-6]. As is evident from this classification system, the residues that are most critical for PDZ domain recognition are P^0 and P^{-2} [6]. This is due to the nature of the interaction between the peptide ligand and the binding site of the PDZ domain.

The ligand binding site of the domain is located in the groove between the βB and αB, and it contains a highly conserved R/K-XXX-G-ϕ-G-ϕ motif [7]. Ligands bind in the extended groove between the βB and αB resulting in a "β-strand addition" to the pre-existing β-sheet of the PDZ domain [8]. The positioning of the peptide ligand is such that the side chains of residues P^0 and P^{-2} point directly into the base of the peptide binding groove. Hydrophobic residues in the domain (typically V, I or L) create a hydrophobic binding pocket in the β-sheet and α-helix groove [9]. The terminal hydrophobic amino acid of the ligand sits in this hydrophobic cavity and mutation of this residue to a hydrophilic amino acid is sufficient to prevent ligand-domain interactions [1, 10]. In fact, the ease in which PDZ domain specificity can be altered by single substitutions is a hallmark of PDZ interactions. Class I domains contain a histidine in the area of the binding pocket that interacts with P^{-2}, thus favoring serine or threonine residues at that position. In class II binding pockets on the other hand, the histidine is replaced by a hydrophobic amino acid, resulting in a preference for hydrophobic residues at the P^{-2} C-terminal position [11, 12]. PDZ interactions are likely fine-tuned by the specific interactions of residues other than P^{-2}

and P^0 with amino acids adjacent to the binding groove, thus allowing for specificity between PDZ targets [7, 13]. While most PDZ ligands described to date are at the extreme C-terminus of the target, internal PDZ motifs have been reported. Internal motifs can be recognized if they form a tertiary structure that mimic a C-terminus. For example, PSD95 can interact with nNOS via a 30 residue section of the nNOS PDZ domain. The 30-residue ligand adopts an extended β-hairpin fold, where the normally required C-terminus is replaced by a sharp β-turn [14].

Studies have reported PDZ-peptide interactions to have binding affinities in the low micromolar range (1-10 μM) [15, 16]. These affinities are similar to those of SH2 and SH3 protein-protein interaction domains; moderate affinities such as these are likely beneficial in regulatory functions where strong but transient interactions are necessary. As mentioned above, one of the most noteworthy aspects of PDZ proteins is that several members contain multiple PDZ domains in tandem, a characteristic mostly absent in other protein-protein interaction domain families. It is of no surprise that these multiple PDZ domain containing proteins often serve as scaffolds for multi-protein signaling complexes.

One example of PDZ proteins functioning as scaffolds is the family of membrane-associated guanylate kinases, or MAGUKs. MAGUKs typically contain 1-3 PDZ domains along with an SRC homology 3 domain (SH3) and a guanylate kinase homology domain (GuK). Through these multiple interaction domains, MAGUKs assemble multi-protein complexes containing receptors, adhesion proteins and various signaling molecules crucial for proper development of the neuronal synapse [17]. The *Drosophila* protein INAD contains 5 tandem PDZ domains and is believed to function as a scaffold for G-protein mediated photo-transduction in the eye. INAD interacts with several proteins in the cascade and mutation of INAD results in the mislocalization of these proteins and loss of signaling [18, 19].

Aside from multi-protein complex formation, PDZ-containing proteins also play a role in the transportation and localization of proteins. One example is NHERF1, which has been shown to regulate membrane protein activity and trafficking (discussed further in following sections). In *C. elegans*, LIN-2, LIN-7 and LIN-10 are PDZ domain containing proteins that form a trimeric complex that regulates localization of certain targets. Mutation of LIN-7 results in loss of LET23 (EGFR homolog) membrane localization, and replacement of the binding interaction domain of LIN-7 and LET23 with a heterologous binding pair rescues the phenotype [20, 21].

Overall, several lines of research over the past couple of decades have demonstrated the importance of PDZ interactions. PDZ domain containing proteins interact with a wide array of proteins, including G-protein coupled receptors (GPCRs), receptor tyrosine kinases, as well as ion channels and transporters. Several proteins discussed throughout this thesis partake in PDZ protein-protein interactions and determining the role of PDZ domains in the regulation of cell signaling is a constant theme throughout the aims presented in this document.

1.2 THE PDZ PROTEIN NHERF1

The Na+/H+ exchanger regulating factor 1 (NHERF1), also known as the ezrin binding phosphoprotein of 50 kDa (EBP50), is a PDZ domain containing scaffold abundantly expressed in the vast majority of epithelial tissues. NHERF1 was originally identified as an essential cofactor for the inhibition of the Na^+/H^+ exchanger 3 (NHE3) by protein kinase A (PKA) in the

kidney [22]. Independent of this observation, another group identified NHERF1 as a 50 kDa protein that binds ezrin with high affinity (hence the name EBP50) [23]. Initial reports identified NHERF1 as a regulator of the localization, signaling and traffic of GPCRs, enzymes, ion channels and transporters [24].

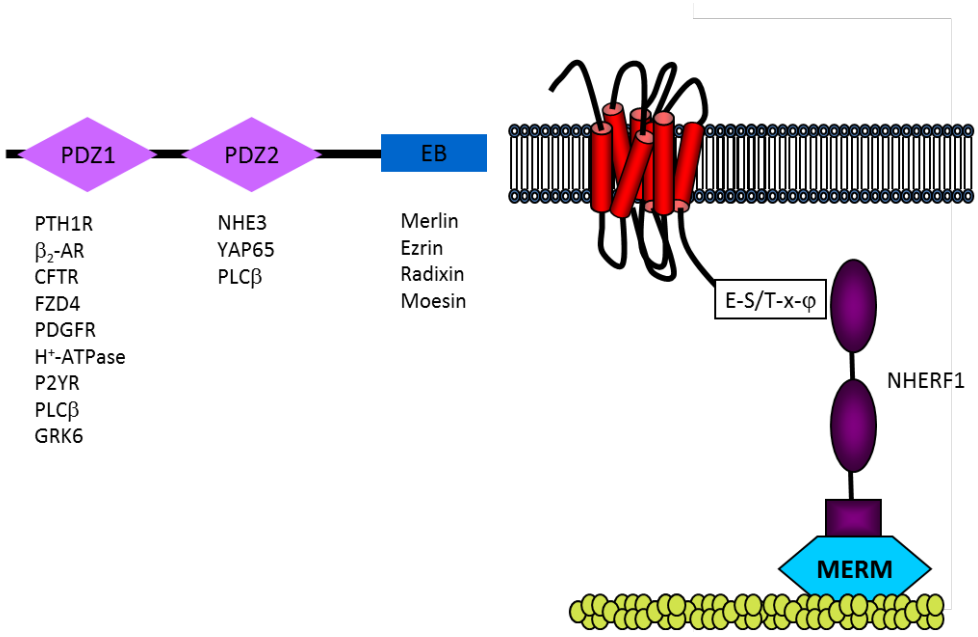

Figure 1. NHERF1 structure and function. NHERF1 contains two tandem PDZ protein-protein interaction domains and a C-terminal domain that bind to members of the MERM family (merlin, ezrin, radixin and moesin). LEFT: diagram of NHERF1 with examples of PDZ-1 and PDZ-2 binding targets. RIGHT: NHERF1 typically binds to targets at the plasma membrane and tethers them to the cytoskeleton via its interaction with ezrin proteins.

1.2.1 NHERF1 function at the cell membrane and cytoskeleton

NHERF1 contains a C-terminal ezrin binding domain (EBD) and two tandem PDZ domains through which it carries out its function (see Fig. 1). Both of NHERF1's PDZ domains are class I PDZ domains that contain a GYGF binding motif. Most of NHERF1's targets interact with the first PDZ domain (PDZ-1) and only a few proteins (such as NHE3 and β-catenin) preferentially

interact with the second PDZ domain (PDZ-2) [25-27]. Over 30 different proteins have been shown to interact with NHERF1, including GPCRs such as the β2-adrenergic receptor (β2-AR), the parathyroid hormone type 1 receptor (PTH1R), and the κ-opioid receptor [24]. Additionally, NHERF1 binds to ion channels, such as the cystic fibrosis transmembrane conductance regulator (CFTR), and growth factor receptors such as the EGFR and the platelet-derived growth factor receptor (PDGFR)[24].

One of the major proteins NHERF1 interacts with is ezrin, a cytoplasmic protein that serves as an intermediate between the plasma membrane and the actin cytoskeleton. Both proteins co-localize at the apical membrane of epithelial cells, where they reciprocally stabilize one another [28]. Studies have demonstrated that NHERF1 and ezrin deficient animals show similar defects in their apical intestinal brush border membrane, implicating NHERF1 and ezrin in maintaining the apical structure of polarized epithelial cells [28]. Along these lines, NHERF1 is believed to function in the stabilization of transmembrane receptors and ion channels at the plasma membrane by anchoring/localizing them to the cytoskeleton [29]. Indeed, NHERF1 expression is thought to increase CFTR conductance by tethering two channels together through the tandem PDZ domains, inducing an activating conformational change [30, 31]. Furthermore, the apical localization of CFTR and the inward rectifier K+ channel ROMK (renal outer medullary potassium channel) is dependent on NHERF1 expression [32]. However, studies have demonstrated that several NHERF1 targets can retain their apical localization even in the absence of NHERF1 expression, suggesting that some of the localization functions of NHERF1 are part of a general and redundant role for PDZ proteins [5, 33, 34].

Several studies indicate that NHERF1 also plays an important role in the trafficking and regulation of GPCRs. The first line of evidence was the observation that NHERF1 expression is

required for β2-AR mediated activation of NHE3. NHERF1 binds to NHE3 and prevents basal activation through its interaction with PKA [35]. Upon activation of β2-AR, NHERF1 binds to the C-terminus of the receptor presumably out-competing the NHERF1-NHE3 interaction. The interaction between NHERF1 and β2-AR is also required for agonist-induced receptor recycling [34, 35]. NHERF1 can also tether β2AR and PTH1R receptors to the actin cytoskeleton and regulate their distribution [36]. Additionally, NHERF1 inhibits ligand-dependent internalization and β-arrestin-independent endocytosis of PTH1R [34, 37, 38]. NHERF1 modulation of PTH1R can also increase cyclic AMP (cAMP) and phospholipase C (PLC) activity. The role of NHERF1 in PTH1R regulation is further supported by the observation that NHERF1 knock out mice present severe deficiencies in mineral ion homeostasis and bone density, functions mediated in part by PTH1R [39]. NHERF1 can also bind to the κ opioid receptor and increase its recycling rate, thus preventing agonist induced down-regulation of the receptor. Frizzled receptors, considered "atypical" GPCRs, can also bind to NHERF1 and this interaction attenuates downstream signaling [40].

The interaction with ezrin and other ERM proteins has a regulatory role on NHERF1 conformation. NHERF1 contains a C-terminal PDZ ligand that in the absence of ezrin binds to the PDZ-2 domain of the protein, adopting a head to tail conformation that reduces the binding affinity of both PDZ domains [41-43]. Studies indicate that the intramolecular interactions between PDZ-2 and the EB region compete with PDZ ligands [43]. Ezrin binding displaces the C-terminus from the PDZ-2, thus making both PDZ domains available to bind to their ligands [44]. This method of regulation may serve as a way to ensure NHERF1 assembles protein complexes only when properly localized and attached to the cytoskeleton.

1.2.2 NHERF1 oligomerization and phosphorylation

Several studies indicate that NHERF1 can form homodimers. Two mechanisms of NHERF1 oligomerization have been observed: head-to-tail dimerization (mimicking NHERF1's own intramolecular regulation) and dimerization of the PDZ domains [41, 45]. It is unclear if NHERF1 dimerization occurs *in vivo* and the precise biological significance of this phenomenon is not fully understood [46]. Dimerization of NHERF1 can be regulated by post translational modifications.

NHERF1 is a phospho-protein that contains 31 S/T residues and phosphorylation of NHERF1 can modulate its binding affinity for certain targets. Although purified PKA does not phosphorylate either recombinant NHER1 PDZ domains or full length NHERF1 directly, PKA induces the phosphorylation of NHERF1 *in vivo* through increased cAMP concentrations [47]. Phosphorylation of the S287, S289 and S290 cluster between PDZ-2 and the EBM by PKA is required for NHERF1 biological activity [48]. NHERF1 is constitutively phosphorylated at S289 by GRK6. Phosphorylation at S289 in rabbit (S290 in humans) is necessary for the assembly of NHERF1 oligomers. S77 is located in the PDZ-1 domain and phosphorylation of this residue by protein kinase C (PKC) significantly reduces its binding to the β2-AR and the sodium-phosphate cotransporter type IIa [47, 49]. PKC phosphorylation of S162 in the PDZ-2 domain attenuates NHERF1 binding to CFTR [50]. Furthermore, phosphorylation by PKC at S339 and S340 can disrupt the head-to-tail autoinhibitory conformation of NHERF1 [42].

1.3 WNT CANONICAL SIGNALING

The Wnt signaling pathway is a highly conserved signaling pathway that regulates several crucial aspects of embryonic development as well as adult tissue homeostasis [51, 52]. Comparative genomics revealed Wnt genes can be found in all metazoan organisms, underscoring their importance throughout animal evolution [53]. The first two members of the Wnt family of genes to be identified were the *Drosophila* gene *wingless (wg*), and its vertebrate homolog *integrated*, or *int-1*. In 1980, genetic studies in *Drosophila* revealed that mutations in the *wg* locus resulted in embryonic lethality and patterning defects, identifying the gene as a major player in embryonic development [54, 55]. A few years later, Nusse and Varmus discovered the preferential integration site for the Mouse Mammary Tumor Virus (MMTV) in breast tumors, and therefore named the gene *integrated* or *int-1* [54]. In 1987 *wg* was shown to be a homolog *int-1* [56], and shortly thereafter the names of the two genes were fused to "Wnt".

Over the past few decades a large body of research has demonstrated that Wnt genes regulate a long list of biological processes such as: cell determination, proliferation, motility, polarity, stem cell renewal, primary axis formation and organogenesis. Deregulated Wnt signaling often results in fatal developmental defects, and in adults can lead to cancer of the breast, colon and skin [52, 57]. There are currently three different signaling pathways that are believed to be regulated by Wnt family members: the canonical Wnt/β-catenin pathway, the non-canonical planar cell polarity pathway (PCP), and the non-canonical Wnt/Ca^{2+} pathway. As the name would suggest, the "canonical" Wnt signaling pathway, which regulates the transcriptional co-activator β-catenin, is the best understood of the three and is the primary focus of the following section.

1.3.1 Wnt ligand production and secretion

Wnt family members are defined by amino acid sequence rather than functional properties. In mammals there are 19 different secreted Wnt proteins. These proteins are typically 350-400 amino acids long and are characterized by a highly conserved cysteine rich region. Despite the presence of an N-terminal signal sequence which targets the protein for secretion, Wnt ligands are virtually insoluble. This unexpected observation is due to the fact that Wnts are palmitoylated at a conserved cysteine that is essential for protein function [58].

The *Drosophila* gene *porcupine (por)* is believed to be responsible for the palmitoylation of Wnt ligands due to it sequence homology with acyl-transferases [59]. This is supported by the observation *por* is required in Wnt producing cells but not cells that receive Wnt signals [60]. In 2006, genetic studies in *Drosophila* revealed that secretion of Wnt proteins depended on a gene named *wntless (wls)*, also known as *evenness interrupted (evi*) [61-63]. The *wntless* encoded protein was found to be primarily localized in the Golgi, and loss of expression retains Wnt ligands inside the cell that produces them. Wnt ligand secretion also requires the retromer complex, a highly evolutionary conserved multi-protein complex involved in intracellular trafficking [62].

Tissue staining demonstrates that Wnts form long-range gradients and are believed to function as morphogens, meaning that their signaling is long range and concentration dependent [64, 65]. How these gradients are formed and regulated is not fully understood. Cytonemes (long and thin filopodial processes) have been speculated to carry Wnts away from signaling cells. One other possibility is that palmitoylation of Wnts limits diffusion from membranes and lipid particles, tethering Wnts to intercellular transport vesicles and lipoproteins [66]. Additionally,

evidence suggests that heparin sulfate proteoglycans (HSPG) may stabilize and transport of secreted Wnt proteins in *Drosophila [67]*.

1.3.2 Wnt receptors and inhibitors

In 1996 Frizzled (Fz) was identified as the receptor for Wnt ligands and in humans ten members of this family of proteins have been identified. Fz receptors possess seven transmembrane domains and long amino-terminal cysteine-rich domain (CRD). Interestingly, Fz receptors contain both internal and carboxy terminal PDZ ligands. Wnt ligands bind directly to the CRD domain of Fz with high affinity [68, 69]. Studies in *Drosophila* and cell culture suggest that Fz receptor activation during canonical signaling is ligand-dependent, and overexpression of receptor in the absence of ligand is insufficient for signaling activation [68, 70]. In addition to Fz, Wnt ligand induced activation also relies on *arrow* in *Drosophila* and low-density-lipoprotein-related protein 5 and 6 (LRP5/6) in vertebrates, both single pass transmembrane receptors required for canonical Wnt signaling. In vertebrates Wnt, Fz and LRP have been reported to form a ternary complex, suggesting that Wnt ligands may physically mediate the interaction between Fz and LRP5/6 [71]. In addition to Wnt, the protein Norrin, which shares no sequence homology with Wnt ligands, can bind to Fz4 and LRP5/6 to promote canonical Wnt signaling [72].

Wnt signaling is antagonized extracellularly through a diverse group of Wnt inhibitors: secreted Frizzled-Related Protein (sFRPs), Wnt-inhibitory factor-1 (WIF-1), and the Dickkopf (Dkk) family of inhibitors are just a few examples [51]. The Dkk family of Wnt inhibitors binds to and antagonizes LRP5/6 receptors and are considered specific inhibitors of Wnt/β-catenin signaling. Although some evidence suggests Dkk1 can induce the internalization and degradation

of LRP5/6 through its interaction with Kremen proteins [73], the currently more accepted model of Dkk1 action is that it disrupts the formation of the Wnt-induced Fz-LRP5/6 complex [74, 75]. SOST, another LRP5/6 antagonist, is also believed to function through this mechanism [76].

WIF and sFRPs bind to Wnt and, in the case of sFRPs, also to Fz, thereby antagonizing both canonical and non-canonical Wnt signaling. Genetic studies in mice indicate significant redundancy of sFRPs [77], and some sFRPs have been shown to possess Wnt-independent activity such as regulation of axon guidance and proteinase inhibition [78]. Shisha proteins are a distinct family of Wnt antagonists that trap Fz protein in the endoplasmic reticulum and prevent localization of the receptor to the cell surface [79].

1.3.3 Wnt deactivated state

In the absence of Wnt, β-catenin is negatively regulated by glycogen synthase kinase 3 (GSK3) and casein kinase I α (CK1α), resulting in reduced β-catenin stability. GSK3 does not bind to β-catenin directly and requires the expression of the protein Axin and *adenomatous polyposis coli* gene products (APC), which along with CK1, are now known as the "β-catenin destruction complex". APC and Axin bind to β-catenin, GSK3 and CK1, promoting their interaction and subsequent phosphorylation of β-catenin [51]. The phosphorylation of β-catenin happens in a sequential manner; first CK1 phosphorylates at the single site S45, and then GSK3 phosphorylates β-catenin at T41, S37, and S334 [52]. N-terminal phosphorylation of β-catenin promotes its interaction with β-TrCP/Slimb, a component of the SCF ubiquitin ligase complex. This ultimately leads to the ubiquitination of β-catenin and its subsequent degradation by the proteasome [80].

Axin and APC are both tumor suppressors critical for β-catenin regulation. Axin is thought to act as a scaffold, and it is the only protein that can interact with all other members of the destruction complex [81]. Additionally, quantitative analysis suggests that the association of Axin is the rate limiting step in the assembly of the destruction complex due to its low levels of expression compared to other members [81, 82].

The precise role of APC in β-catenin regulation remains uncertain. Studies have demonstrated that both APC and Axin can be phosphorylated by GSK3 and CK1, and these modifications lead to increased affinity for the same domain of β-catenin [80, 81]. This observation led to the hypothesis that APC functions by removing phosphorylated β-catenin from Axin, increasing β-catenin turnover and allowing Axin to participate in another round of β-catenin phosphorylation [80]. Consistent with this model, overexpression of Axin in cancer cells that do not express APC restores β-catenin inhibition, suggesting that APC is only necessary when Axin levels are low.

Interestingly, APC can actually promote Wnt signaling *in vivo* by enhancing Axin degradation, a function dependent on the N-terminal portion of APC which is not involved in β-catenin degradation [81, 83]. One explanation for this paradoxical observation is that it helps buffer dramatic changes in β-catenin when APC levels vary; a decrease in APC would then result in increased Axin levels, thereby compensating for decreased β-catenin degradation [81]. Colon cancer cells often retain the N-terminal half of APC and could potentially use this portion to further enhance β-catenin signaling [84-86]. APC has also been shown to function in the nucleus to promote β-catenin nuclear export and act as a suppressor for β-catenin target genes [87].

Further regulation of the destruction complex can be mediated by two S/T phosphatases, PP1 and PP2A. PP1 dephosphorylates Axin, promoting the dissociation of the Axin complex

[88]. PP2A can dephosphorylate β-catenin, and evidence suggests APC can protect β-catenin from its activity [89]. How the activity of these phosphatases is regulated by Wnt signals has yet to be determined.

1.3.4 Wnt activated state

Wnt-mediated activation of β-catenin occurs after Wnt ligands bind simultaneously to Frizzled and the co-receptors LRP5/6. This is hypothesized to result in the formation of large protein complexes dubbed "signalosomes" at the plasma membrane, which are believed to be the critical first step in subsequent Wnt signaling transduction.

According to the signalosome hypothesis, ligand binding to Fz and LRP receptors promotes a clustering of these receptors that recruits the cytoplasmic phosphoprotein Dishevelled (Dvl) to the plasma membrane [90]. Dvl is a PDZ domain containing protein; surprisingly the Fz-Dvl interaction is mediated by an internal PDZ ligand, despite the presence of C-terminal PDZ ligands in 8 of the 10 human Frizzled receptors [91]. Dvl also contains a highly conserved DIX domain that it shares with the protein Axin, and this domain mediates a reversible head-to-tail polymerization crucial to Dvl signaling activity [92]. Wnt activation of receptors triggers the polymerization of Dvl [93]. Polymerization of Dvl allows the protein to co-polymerize with Axin via their shared DIX domains, thus recruiting it (and associated proteins) to the plasma membrane to form part of the signalosome [94]. Further signaling requires the phosphorylation of LRP5/6.

LRP5/6 receptors contain five repeats of PPPSPxS motifs, which upon phosphorylation can serve as docking sites for the Axin complex. Remarkably, knock out studies revealed that the main

kinase responsible for LRP6 phosphorylation at PPSP sites is Axin-bound GSK3 [95, 96], making GSK3 both a positive and negative regulator of β-catenin signaling.

Once the PPSP motifs of LRP5/6 have been phosphorylated, this primes the receptor for further phosphorylation. Clustered Dvl promotes phosphorylation of LRP6 by CK1γ and CK1ε at adjacent S sites (PPPSPx**S**) [97], which is crucial for the functioning of the signalosome [90, 96]. It is interesting to note that this phosphorylation sequence is the opposite of the one for β-catenin, where CKIα primes the protein for GSK3 phosphorylation. Once LRP6 is fully phosphorylated the PPPSPXS sites serve as loading docks for Axin, which recruits the β-catenin destruction complex [98]. The fact that Axin is recruited by Dvl and required for LRP phosphorylation, and that in turn LRP phosphorylation recruits Axin, suggests a positive feed-forward loop in Wnt/β-catenin signaling that ensures phosphorylation of all five PPPSPxS motifs. This idea is supported by the observation that LRP6 activity is particularly sensitive to PPPSPxS copy number [99].

How signalosomes inhibit phosphorylation of β-catenin by GSK3 is not fully understood. In 2006, Mi and colleagues reported that GSK3 can bind directly to the cytoplasmic tail of LRP6 and phosphorylate it [100]. Shortly after this discovery, it was reported that phosphorylation of LRP6 at PPPSPxS motifs is crucial for GSK3 inhibition [101]. Additionally, dually phosphorylated PPPSPxT peptides were shown to be sufficient to inhibit GSK3 kinase activity towards β-catenin and other targets *in vitro* and *in vivo* [102, 103]. Taken together, these observations suggest that the phosphorylated LRP motifs may act as pseudo-substrates for GSK3, competitively inhibiting its interaction with β-catenin. This particular model also fits well with previous observations of GSK3 regulation by Akt, which phosphorylates GSK3 at the N-terminus, creating a pseudo-substrate (phospho-S9-GSK3) that is thought to fold back into the catalytic pocket of GSK3 [104].

A fairly recent study published in 2010 proposes a different model describing how Wnt canonical signaling may decrease GSK3 activity through the sequestration of the enzyme from its substrates in internal membranes [105]. The study demonstrates that Wnt canonical signaling activity results in internalization of GSK3β (and other components of the signalosome) in multivesicular bodies (MVBs). Consequently, GSK3 can no longer interact with newly synthesized β-catenin. This is supported by the observation that cell extracts solubilized with Triton X-100 did not contain Wnt-inhibited GSK3, while cell extracts solubilized with digitonin (which does not solubilize intracellular vesicles) showed a Wnt induced 66% decrease in GSK3 activity. However, an alternative explanation is that Triton X-100 may have a binding affinity to the signalosome complex itself. Furthermore, the authors were unable to detect a reduction in endogenous GSK3β, which one would expect if the enzyme was internalized in MVBs, which eventually fuse with lysosomes [106].

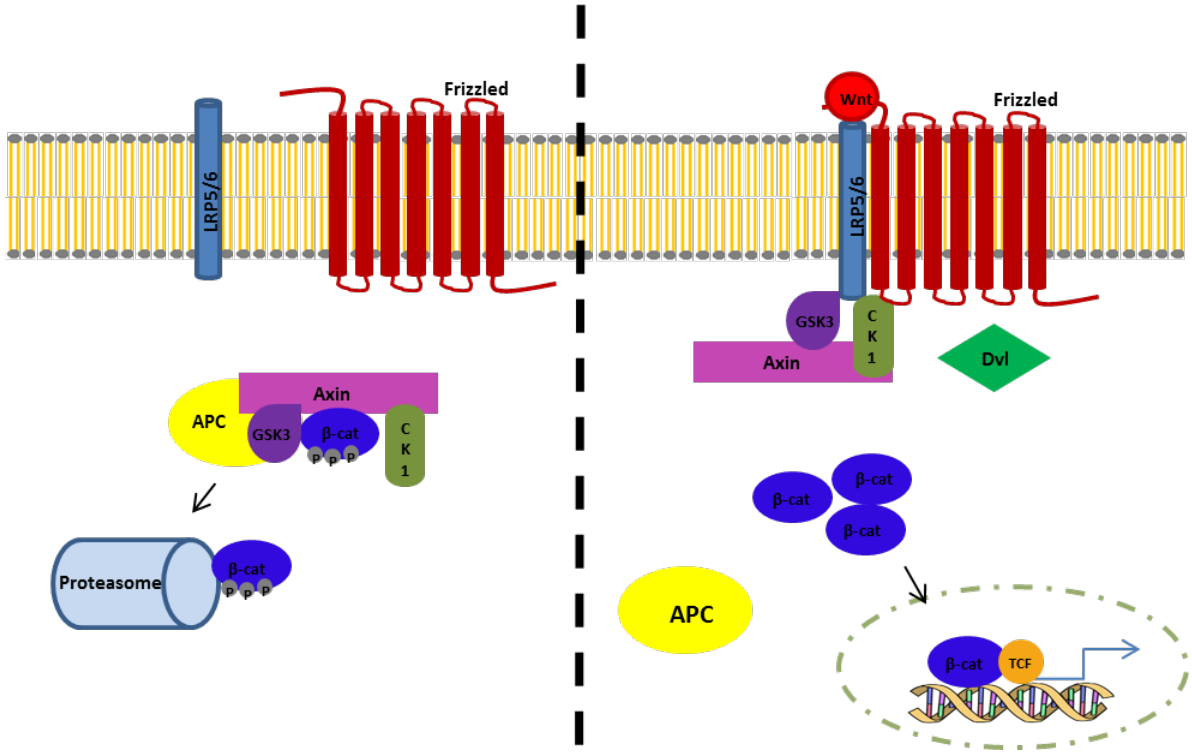

Figure 2. Wnt Canonical Signaling. In the absence of Wnt ligands, the N-terminus of β-catenin is hyper-phosphorylated by the β-catenin destruction complex, which targets the protein for proteasome degradation. The destruction complex is composed of the scaffold protein Axin, the tumor suppressor APC, and the kinases CK1 and GSK3. In the presence of Wnt ligands, Wnt binding increases the association of Fzd and LRP5/6 receptors, which recruits the cytoplasmic protein Dvl. Dvl polymerization induces GSK3 and CK1 dependent phosphorylation of LRP5/6, which results in decreased degradation of β-catenin and increased β-catenin nuclear localization. Once in the nucleus, β-catenin binds to TCF transcription factors to regulate TCF/LEF target genes.

1.3.5 β-catenin in the nucleus

The decreased phosphorylation of β-catenin following canonical Wnt signaling activation leads to increased stability of the protein and translocation to the nucleus. The precise mechanism by which β-catenin is shuttled to the nucleus remains unknown. β-catenin nuclear import does not depend on the Nuclear Localization Signal/importin machinery, and it has been proposed that β-catenin enters the nucleus through direct interaction with nuclear pores [107]. Some evidence suggests that two proteins, TCF and Pygopus, can promote β-catenin nuclear localization, however β-catenin can

still localize in the nucleus in the absence of either protein [108]. β-catenin can also be exported from the nucleus by APC or Axin [107, 109].

Once in the nucleus, β-catenin forms complexes with members of the TCF/LEF family of transcription factors. Vertebrates have four TCF/LEF genes that differ dramatically in their sequence, yet are biochemically similar and were found to be largely redundant in knockdown studies [110]. In the absence of Wnt, TCF associates with Groucho (TLE1 in humans), which promotes chromatin compaction to repress Wnt target genes [111]. Upon Wnt activation, β-catenin binds to TCF, displacing Groucho/TLE1 and recruiting the histone acetylase and co-transcriptional factor CBP/p300.

The association of β-catenin to TCF is thought to create a bipartite transcription factor in which TCF is the DNA binding domain and the C terminus of β-catenin is the transactivation domain. β-catenin and TCF bind to DNA consensus sequences CCTTGWW (W represents either T or A), referred to as Wnt responsive elements (WREs), inducing DNA bending and chromatin remodeling conducive to transcriptional activation. The nuclear antagonists Chibby and ICAT bind to β-catenin and disrupt β-catenin/TCF and β-catenin/co-activator complexes, resulting in increased nuclear export of β-catenin [112, 113]. Additionally, TCF/LEF transcription factors can be inhibited through post-translational modifications including phosphorylation, acetylation, sumoylation and ubiquitination.

1.3.6 Wnt signaling in cancer

Many proto-oncogenes encode components of growth signaling pathways. β-catenin transcriptional activation produces changes in gene expression that can result in transformation and

loss of epithelial phenotype. Some of these targeted genes are developmental regulatory genes such as *siamois, twin* and *Xnr-3* in *Xenopus.* Additional targets include regulators of cell growth and proliferation: *c-myc* and *cyclin D1* [114, 115]. *C-myc* is a powerful oncogene that promotes cell cycle progression by inducing G1/S phase transition and its expression is promoted by Wnt ligands. DNA microarray analysis revealed several other genes regulated by β-catenin/TCF that are implicated in the proliferation and differentiation of cells, including: *c-myb, ets, bmp4* and ephrin receptors EPHB2 and EPHB3 [116]. Studies in colorectal carcinomas confirm the generalization that β-catenin/TCF targets often work to repress differentiation and induce proliferation [117].

Wnt signaling deregulation in colorectal cancer has been well documented. The first piece of evidence was the discovery that germline mutations in APC were the cause of Familiar Adenomatous Polyposis (FAP) in 1991. FAP is a hereditary cancer characterized by the development of a large number of colon polyps early in life [118, 119]. While FAP patients inherit only one mutant APC allele, eventually loss of the second allele occurs in some cells leading to the development of malignant carcinomas. The role of APC as a tumor suppressor in the colon was further supported by studies that demonstrated that a vast majority of spontaneous colorectal cancers lose both APC alleles [120]. Over 80% of colorectal cancers harbor mutations for APC, and over 300 different disease associated mutations of APC have been identified [85]. Inactivating mutations of APC increase β-catenin stabilization and protein levels [121]. In the small percentage of colon cancers without APC mutations, mutations in other members of the Wnt/β-catenin signaling pathway have been found: mutations in Axin2 [122], and activating mutations of β-catenin [84].

Activating mutations of the Wnt pathway can also be found in other types of sporadic tumors. Many upper gastrointestinal tumors contain mutations of β-catenin and APC genes [123-126]. Furthermore, deregulation of Wnt signaling has been implicated in the development of hair follicle tumors, or pilomatricomas. Several studies have reported activating mutations of β-catenin in the majority of spontaneously occurring human pilomatricomas [127-129]. Transgenic mice that express stabilized β-catenin develop lesions similar to human epithelial cysts and pilomatricomas [130].

Wnt signaling has also been implicated in the development of hepatocellular carcinoma (HCC), which accounts for 75% of all liver cancers [131]. Indeed, one of the first studies suggesting the role of Axin as a tumor suppressor came from a report that demonstrated inactivating mutations of *AXIN1* in HCC [132]. Several studies have looked at β-catenin gene (*CTNNB1*) mutations in HCC; collectively, these reports show that approximately 20% of HCC carcinomas harbor activating mutations of β-catenin [132-137]. One study suggests that mutations in β-catenin are more common in HCCs associated with hepatitis C infections (41%) [138]. Furthermore, the expression of nuclear β-catenin has been demonstrated to be significantly correlated with patient clinical outcome [136].

Reproductive organs are also prone to cancers characterized by upregulated Wnt signaling. Nuclear expression of β-catenin was identified in approximately 30% of endometrial-type ovarian cancers. Moreover, sequence analysis identified *CTNNB1* mutations in 28% of primary tumors [136, 139-143]. Furthermore, mutations in APC and AXIN1/2 have also been reported in endometrial-type ovarian carcinomas [141]. One study also reported increased nuclear β-catenin in 37% of cervical cancers [144]. Finally, three different groups have collectively reported a small (5%) but noteworthy presence of *CTNNB1* mutations in prostate cancer [145-147].

Several studies suggest a role for Wnt canonical signaling in mammary development and carcinogenesis in animal models. One of the first pieces of evidence indicating that deregulated Wnt signaling can lead to carcinogenesis was the observation that mice ectopically expressing Wnt-1 developed mammary tumors [148]. Others have reported amplification of the *Wnt-2* gene in mice mammary tumors [149]. TCF1 knock out mice eventually develop adenocarcinomas in the intestines and mammary glands [150]. Furthermore, transgenic mice with mammary-specific *APC* mutations and mice overexpressing Axin have defects in mammary and lymphoid development [151, 152]. Surprisingly, very few if any mutations of *CTNNB1*, *APC* or *AXIN* genes have been found in human breast cancer [153-155]. One study did report identifying *APC* mutations in 18% of primary breast cancer samples screened, however most of these mutations were downstream of the cluster region for *APC* truncations associated with constitutive activation in colon cancer, so the status of *APC* function in the samples was unknown [156].

Nevertheless, increased nuclear β-catenin has been reported in 60% of human breast cancer samples and it is significantly correlated with poor prognosis [157]. Moreover, some reports indicate overexpression of WNT-2, WNT-5A and WNT 7B ligands in some breast cancer tumors and cell lines [158-161]. Together, these observations indicate that despite the lack of Wnt gene mutations identified in human breast cancer, upregulation of the pathway through ligand overexpression or some other unidentified means could be contributing to mammary carcinogenesis. Our laboratory recently published findings that implicate the PDZ scaffold NHERF1/EBP50 in the regulation of Wnt canonical signaling [40]. NHERF1 is highly expressed in mammary tissue and is regulated by estrogen [162, 163]. NHERF1 and other atypical regulators of Wnt signaling yet to be identified could potentially play a role in the regulation of canonical Wnt signaling in the breast, thus explaining the lack of Wnt mutations identified in breast cancer.

Hopefully, future studies will be able to further elucidate the role of Wnt canonical signaling in human breast cancer.

1.4 WNT PLANAR CELL POLARITY SIGNALING

The Wnt/Planar Cell Polarity pathway is one of the "non-canonical" branches of Wnt signaling and its transduction is independent of β-catenin activity. Planar cell polarity (PCP) refers to the polarization of cells within the plane of the epithelial tissue (perpendicular to the apical-basal axis), and its regulation differs from that of apical-basal polarity [164, 165]. Examples of PCP include the uniform alignment in proximal-to-distal orientation of the bristles at the apical surface of wing cells in *Drosophila*, and the uniform medial-to-lateral orientation of stereociliary bundles in the Corti organ (a mammalian auditory sensory organ). PCP is essential for a variety of developmental events involving cell fate decisions, morphogenesis and organized cell movement.

Wnt/PCP signaling has been shown to be critical for several processes in development, including gastrulation, neural tube closure, as well as ear patterning and hearing. Despite the importance of PCP, we know very little about this signaling pathway (compared to the breadth of information available on Wnt/β-catenin signaling). Most of what we know about PCP signaling is through the manipulation of gene expression in animal models. However, the underlying molecular mechanism by which Wnt PCP family members transduce their signal remains largely enigmatic.

1.4.1 Wnt PCP signaling in development

Extensive genetic studies in *Drosophila* identified a set of six genes indispensable for proper PCP in adult fly tissues: Frizzled (Fz), Flamingo (Fmi,), Strabismus (Stbm, also known as Van Gogh or Vang), Prickle (Pk), Dishevelled (Dsh,) and Diego (Dgo). Fz, StbM and Fmi are transmembrane proteins; their vertebrate homologs are Fzd, Vangl (Van Gogh-like) and Celsr (Cadherin EGF LAG Seven-Pass G-Type Receptor 1) respectively; of these receptors Vangl and Fz contain class I PDZ ligands that can potentially interact with NHERF1. Prickle, Dsh (Dvl in vertebrates) and Dgo (Dishevelled associated activator of morphogenesis, Daam1, in vertebrates) are cytoplasmic proteins. These proteins are thought to be the "core" PCP signaling components [164, 166]. Two of these components, Fz and Dsh, are shared with the canonical Wnt pathway. In *Drosophila* the proteins Inturned, Fuzzy and Fritz have been implicated as downstream effectors of PCP signaling [166, 167]. Mutation of any of these genes results in disorganized bristle and hair growth. Interestingly, Wnt ligands were not found to be necessary for PCP in *Drosophila*, yet "non-canonical" Wnt ligands have been implicated in PCP signaling in vertebrates [168, 169].

Studies in *Drosophila* lead to the observation that PCP molecular events often begin with the asymmetric localization of Fz, Vang and Dsh proteins at the cell surface. How PCP is initiated is still not fully understood. One hypothesis states that the first directional cue for polarization is regulated by the atypical cadherins Fat (Ft) and Dachsous (Ds), which are thought to act together with the Golgi protein Four-jointed to polarize Fz signaling [170]. Alternatively, others have proposed that an unidentified morphogen acts in parallel to Fj, Ds and Ft to localize the PCP receptors [171]. The fact that Wnt ligands (Wnt5a and Wnt11) are necessary to mediate

certain PCP events in mice also supports the idea that a morphogen gradient may act to localize the core PCP receptors. Nevertheless, Ft and Ds asymmetric localization appears to induce microtubule alignment in a proximal-distal orientation at the apical surface of the cell [171, 172].

Asymmetric distribution of Wnt PCP receptors is transient and precedes major morphologic events. In the fly, Vang-Pk complexes localize in the proximal side of the cell and Fz, Dsh and Dgo proteins localize distally immediately preceding the initiation of hair growth in the wing [173]. The transmembrane protein Fmi is localized both proximally and distally during initial PCP events and is thought to mediate PCP by participating in homophilic interactions between cells (where one adhesion molecule binds to another of the same kind) and participating in the asymmetric localization of Vang and Fz, thereby communicating the PCP signal between the cells in a tissue [174]. Additionally, Vang has been reported to interact with Fz heterophilically between cells, providing further communication between cells on the tissue plane [175].

Wnt/PCP signaling has been demonstrated to be crucial for proper convergent extension in vertebrates. Convergent extension (CE) is a morphogenetic process by which cells "crawl" between one another, forming a long narrow array. This process is thought to be mediated by polarized lamelipodia (actin-rich protrusions that generate traction for cell movement) [176, 177]. In *Xenopus*, the loss of Dvl results in the randomized orientation of lamelipodia, resulting in failed CE [178]. Proper CE is crucial for spinal cord closure and mutation of PCP components in mice often lead to tube closure defects. For example, the classical mutation of Vangl in mice, *Looptail*, results in defective neural tube closure and a "looped tail" appearance [179, 180]. In 2002, time lapse studies in *Xenopus* demonstrated that PCP dependent CE was required to close the distance between the neural folds of the spinal cord [178]. Shortly after this discovery, Dvl,

Celsr, and Fz were also reported to be necessary for proper closure of the neural tube in mice [181, 182].

Wnt PCP signaling is also critical in organogenesis. Expression of Vangl1, Vangl2, Dvl2 and Dvl3 are all critical in heart looping and chamber formation and loss of any of these proteins leads to cardiac abnormalities [183-185]. Mutation or loss of *Wnt5a* and *Vangl2* alleles leads to defects in the formation of the forestomach in mice [186]. *Looptail* Vangl2 mutant mice also present defects in the uterus and vagina, including: short uterine horns, failure of uterine horns to fuse at the cervix, and imperforate or septate vaginas [187]. Additionally, loss of Wnt9a leads to formation of kidney tubules with abnormally large diameters and eventual kidney cyst formation [188]. Furthermore, Celsr1 and Vangl2 are required for the proper branching in the mouse lung and kidney [189, 190].

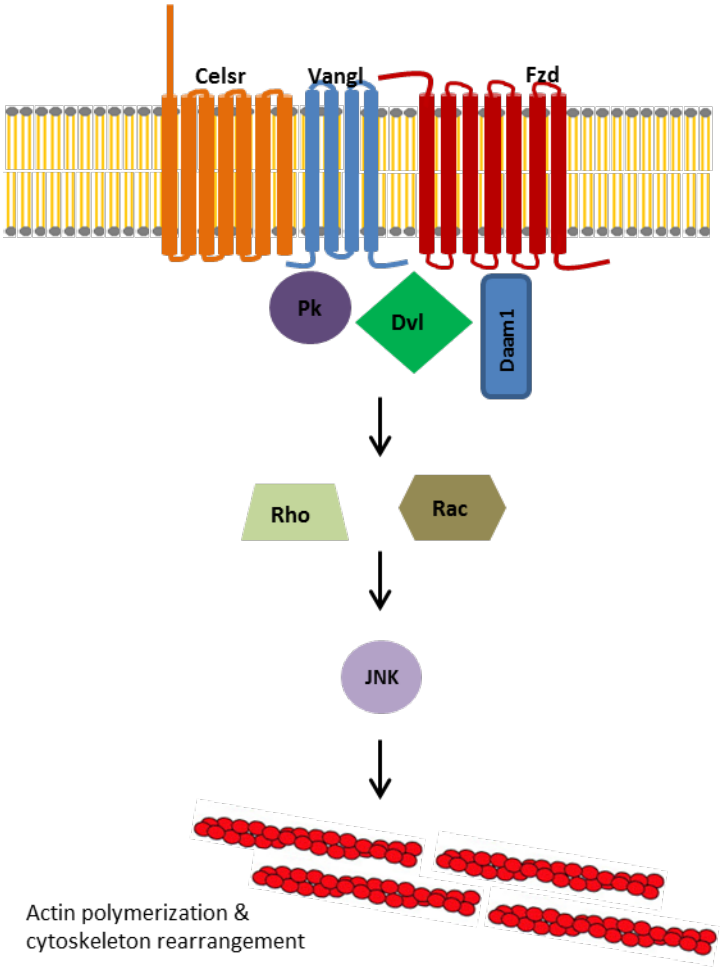

Figure 3. Wnt Planar Cell Polarity signaling. Extensive genetic studies have identified the following core essential components of Wnt planar cell polarity signaling: Fzd, Vangl, Dvl, Daam1, Celsr and Pk. Expression of these proteins leads to downstream activation of Rho/Rac GTPases and JNK, resulting in actin polymerization and cytoskeletal rearrangement.

1.4.2 Wnt/PCP signaling in ciliary function

Cilia are organelles that project from almost all animal cells and their functions include: mechanosensation by primary cilia and the induction of directional flow of fluid by motile cilia. Ciliopathies include polycystic kidney disease, Bardet-Bidel syndrome (BBS), Meckel-Gruder syndrome, and orofaciodigital syndrome amongst others [191]. One of the first indications that

PCP signaling could play a role in ciliogenesis was the observation that mice with mutations in genes linked to human BBS, a disorder associated with dysfunction of primary cilia, showed defects in neural tube closure and misalignment of hair cells in the cochlea [191]. Another clue was the similarity in sequence between Dgo/Daam1 and Inversin, a protein critical for cilia function.

Loss of Inversin in zebrafish and *Xenopus* revealed that the protein is also critical for CE. Interestingly, Vangl2 and Dvl both localize near cilia bodies in vertebrate cells [192, 193]. Dvl interacts with Daam1, a formin protein that regulates the formation of actin filaments, to polymerize actin and regulate its distribution. As one may expect, actin filaments are required for the proper positioning of ciliary bodies, and in cells where the actin cytoskeleton is disrupted cilia can be found in the cytoplasm of the cell [194, 195]. Furthermore, loss of Dvl3 in *Xenopus* results in decreased number and size of cilia in multiciliated epithelial cells [196]. Knockdown of the PCP effectors Inturned or Fuzzy in *Xenopus* has been reported to result in reduced ciliogenesis and reduced actin polymerization [193].

In the inner ear, mechanosensory hair cells can be found in the organ of Corti, which is responsible for the recognition and transmission of auditory signals. Mechanosensory hair cells consist of bundles of stereocilia that protrude from the luminal surface of the cell. These bundles must be aligned and oriented in one direction for proper function. In Vangl2 Looptail mutants, hair cell orientation in the organ of Corti is completely randomized, furthermore, Fzd3 expression at the membrane is abrogated in these cells [182, 197]. Mutation of Celsr leads to a similar phenotype in mice characterized by loss of Vangl2 polarization in the organ of Corti [198].

Loss of PCP signaling has been shown to impair the function of motile cilia. More recently Vangl2 has been shown to regulate the posterior tilting and organization of motile cilia. Although Vangl2 mutants can form cilia, the orientation of the cilia is disorganized, resulting in lack of directional flow [199]. Knockdown of Celsr2 & Celsr3 in mice results in impaired ciliogenesis in the ependymal layer of the brain, resulting in a form of communicating hydrocephalus that leads to pathological accumulation of cerebral spinal fluid in the brain [198]. It has been speculated that mutation of other PCP components do not inhibit ciliogenesis due to the inherent redundancy within each protein family, as evident by the fact that single Celsr knockdown causes a much milder hydrocephalus phenotype [198]. Others have reported that cilia-generated flow can feed back to fine tune the direction and orientation of cilia in *Xenopus*, and that Wnt PCP signaling may be involved in this feedback signal [200, 201].

1.5 ESTROGEN SIGNALING AND BREAST CANCER

Estrogen, or 17β-estradiol (also known as E2), is a steroid hormone that is characterized by three conjugated aromatic rings. E2 plays a significant role in reproductive function and mammary gland development. Approximately 70% of all diagnosed breast cancers express the estrogen receptor (ER), and these cancers depend on estrogen for growth and proliferation. Estrogen signaling involves both genomic (nuclear) and non-genomic (extra-nuclear) pathways. In the genomic pathways, ligand bound ER dimers regulate target gene expression through direct interactions with DNA. Non-genomic estrogen signaling involves the rapid and transient

activation of several kinase cascades mediated by translocation of ERs to the cytoplasmic side of the cell membrane.

There are two subtypes of ER, ERα and ERβ. Experimental and clinical evidence suggests that the ERα subtype is the main mediator of signaling for the majority of breast cancers [202, 203]. In this section, I will introduce E2 signaling with emphasis on its role in mammary gland development and breast cancer.

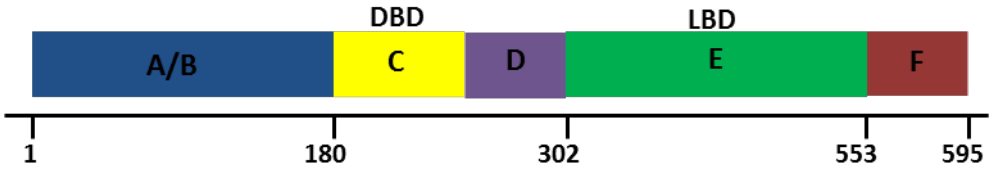

Figure 4. ERα structure. **A/B**: a modulatory N-terminal domain that is also known as the activation function domain 1 (AF1). Domain **C** is a highly conserved DNA binding domain (DBD) located near the center of the receptor. The **D** domain, also known as the "hinge region", is a highly flexible region which contains a nuclear localization signal that becomes exposed upon ligand binding. Following the hinge region is the **E** domain, or the ligand binding domain (LBD), which contains the hormone binding pocket. The C terminal region of ERα contains the **F** domain which is necessary for gene modulation and receptor dimerization. The E and F domains are also known as the second activation function domain (AF2) for their crucial role in modulating ER transcriptional activity.

1.5.1 ER signaling in mammary development

The importance of ERα in the post-natal development of mammary gland was first demonstrated in ER receptor knockout studies in mice [204]. At birth, the mammary gland is underdeveloped. On the onset of puberty, E2 and progesterone initiate the maturation of the mammary gland together [205]. E2 signaling triggers ductal elongation during puberty and knockout of ERα (which mediates E2 actions in mice) results in an undeveloped ductal system that fails to branch out [206]. In ERα null mice, ducts failed to invade into the fat pad beyond the nipple. Evidence

suggests that ERα not only regulates ductal morphogenesis during puberty, but also alveleogenesis during pregnancy and lactation [207, 208]. ERβ knockout mice however show no difference in mammary gland morphology or development when compared to wild-type, indicating that ERα is the ER subtype responsible for regulating mammary gland development [209]. Interestingly, ERβ can inhibit the ERα mediated proliferation of breast cancer cells [210].

1.5.2 ERα genomic signaling

In the absence of ligand, ERα is sequestered in complex with an inhibitory heat shock protein in cell nuclei. Ligand binding induces a conformational change within the ER that induces dissociation from chaperone proteins and promotes homo-dimerization. The ER dimers can bind directly to DNA with high affinity to specific sequences termed estrogen response elements (EREs), which are cis-acting enhancers located within the regulatory regions of target genes [211]. The first ERE was initially identified in the estrogen responsive sequence of the vitellogenin A2 promoter in *Xenopus laevis* [212]. In humans, the ERE sequence is a 15bp palindrome consisting of two GGTCA half sites separated by a 3 bp spacer; however, ERα can also bind to imperfect or half-ERE sites. Binding of the ER to these sequences can either enhance or inhibit the transcriptional activity of the downstream target gene. One study demonstrated that only a small fraction of ERα binding sites are located in the promoter regions but instead are found at relatively long distances from target genes [213].

For genes lacking functional ERE sequences, ER can activate transcription indirectly through protein-protein interactions with other DNA-bound transcription factors. Approximately 35% of human E2-responsive genes are activated via ER-indirect DNA association [214]. Some of the

major co-transcription factors involved in E2 signaling are Stimulating protein-1 (Sp-1), nuclear factor-κ B (NF-κB), and activator protein 1 complex (Ap-1). Sp-1 is responsible for the E2 mediated induction of low-density lipoprotein (LDL) receptor [215], endothelial nitric oxide synthase (eNOS) [216], cyclin D1 [217], and c-Myc amongst many others [218]. Activated ER enhances the binding of Sp-1 to GC-rich sequences in the promoters of target genes, recruiting co-activators and initiating transcription [219]. NF-κB transcription factor regulates genes responsible for inflammation and immune responses. ERα prevents NF-κB stimulated transcription of genes, such as interleukin-6 (IL-6), via its interaction with the c-rel subunit of the NF-κB complex [220]. AP-1 is actually a complex of several proteins (including fos, jun, and others) that regulates genes involved in growth, differentiation and development. AP-1 is required for E2 mediated induction of insulin-like growth factor I (IGF-I), ovalbumin, progesterone receptor, and ps2/TFF1 [218].

Over the years several more co-regulatory proteins for ER have been identified, including co-activators and co-repressors. Co-regulators often contain Leucine rich motifs (LXXLL, where X is any amino acid) that interact with the ligand binding domain of ERα [221]. Most of these co-regulators are associated with various enzymatic properties that regulate chromatin remodeling, such as: acetyltransferases, deacetylases, methyltransferases, phosphokinases, ubiquitin ligases, and ATPases. Therefore, it is generally believed that ERα co-regulators promote ER signaling by stabilizing the formation of transcription initiation complexes [222-224]. For example, CBP and p300 acetylate histones at ERα target gene locations, thereby facilitating chromatin remodeling and recruitment of transcription initiation complexes [224]. The Switch/Sucrose Non-Fermentable (SWI/SNF) chromatin remodeling complex has also been demonstrated to regulate ERα transcription [225].

Many of the ERα target genes are believed to contribute to oncogenesis. Some of the first ERα regulated genes identified in breast cancer cells were *pS2TFF1*, *c-myc*, and *cyclin D1* [226-228]. How pS2/TFF1 contribute to breast cancer pathology is not fully understood, however others have reported that over expression of pS2/TFF1 in MCF-7 cells is associated with increased cell proliferation, migration and motility [229]. Furthermore, pS2 expression is higher in transformed cells positively correlated with ERα status in breast tumor samples [230, 231]. *Cyclin D1* and *c-myc* are powerful oncogenes (also regulated by Wnt/β-catenin signaling as mentioned in the previous sections) that promote cell cycle progression and cell proliferation. Mammary specific overexpression of *cyclin D1* results in the development of mammary carcinomas [232]. ERα can also regulate cell cycle progression by repressing p27, an inhibitor of cyclin-dependent kinase 2 (CDK2), resulting in increased CDK2 activity and G1/S progression [233].

Other ERα target genes implicated in breast cancer include: *FOXM1*, *Efp*, *PELP1*, *CIZ1* and *GREB1* amongst others. FOXM1 plays a key role in cell cycle progression and its expression is increased in the S and G2/M phases of the cell cycle [234]. FOXM1 is believed to mediate mitotic division and is upregulated in breast cancer. Efp (estrogen-responsive finger protein) is an ubiquitin ligase that promotes breast cancer progression by targeting 14-3-3δ for degradation, a protein which inhibits cell cycle progression [235, 236]. PELP1 (proline, glutamic acid, and leucine-rich protein) is a co-activator of ERα believed to increase cell proliferation. CDKN1A-interacting zinc finger protein 1 (Ciz1) is also an ERα co-activator and along with PELP1 is positively regulated by E2 signaling, thus their over expression confers hyper-sensitivity to E2 in breast cancer cells [237-239]. Ciz1 is believed to promote the interaction between ligand bound ERα and target genes. GREB1, or growth regulation by estrogen in breast cancer 1, is another

ERα target gene that increases cell proliferation in breast cancer cells [240]. The metastasis-associated protein 3 (MTA3) promotes epithelial-mesenchymal transition (EMT) and metastasis in breast cancer tumors [241, 242].

Aside from the target genes themselves, several co-regulators of ERα genomic activity are known to be de-regulated in breast cancer. Overexpression of amplified in breast cancer 1 (AIB1), breast carcinoma amplified sequence 3 (BCAS3), MUC1 and several other ER co-activators have been linked to either breast carcinogenesis or tamoxifen resistance [243-246]. AIB1 interacts with ERα in a ligand-dependent manner and aids in the recruitment of histone acetyltransferases such as p300 and P/CAF to target gene chromatin [247]. BCAS3, also recruits p300 and CBP and is dependent on PELP1 function [232]. MUC1 is a transmembrane glycoprotein normally localized on the apical surface of secretory mammary epithelium that also serves as a potent co-activator of ERα signaling. MUC1 binds to the DNA binding domain of ERα stabilizing its expression and blocking its degradation in breast cancer cells [248].

Co-repressors of ERα signaling are also de-regulated in breast cancer. Typically, co-repressors counterbalance the action of ER co-activators by recruiting histone deacetylases (HDAC) and promoting chromatin condensation. Therefore, loss of co-repressor expression promotes breast cancer progression [249]. Repressor of ER activity (REA) and nucleosome remodeling histone deacetylation complex (NuRD) both function as HDACs and their de-regulation results in an invasive cancer phenotype and increased ERα expression [250, 251]. Nuclear receptor corepressor 1 (NCOR1) inhibits ERα by binding to the ligand binding domain of the receptor and reduced expression of NCOR1 is associated with shorter relapse-free periods and decreased survival in breast cancer patients [252]. Furthermore, decreased NCOR1 expression has been implicated in acquired tamoxifen resistance [253].

1.5.3 Non-genomic ER signaling

While the majority of ER is localized in the nucleus, several lines of research have revealed that ER can function outside the nucleus in “non-genomic” ER signaling pathways. Palmitoylation of ERα at cysteine 447 localizes the receptor to the plasma membrane and upon ligand binding activates MAPK and PI3K/AKT signaling cascades in breast cancer cells [254]. Further activation of these pathways can result from E2-mediated activation of various growth factor receptors such as IGF-1 and epidermal growth factor receptor (EGFR) [255]. Studies have reported that inhibition of MAPK signaling results in significantly reduced cell proliferation and tumor growth, suggesting that ERα/MAPK signaling is crucial to breast cancer progression. Similarly, PI3K inhibition by LY294002 blocked ERα mediated breast cancer cell migration [256]. Furthermore, E2 can induce Bcl-2 activity through both the Ras/PI3K/AKT and Ras/ERK/p90RSK1 pathways, thus suppressing apoptosis [257].

Recent evidence suggests that ERα genomic co-regulators can also regulate non-genomic signaling. PELP1, for example, can promote the interaction of ERα and Src, resulting in E2 mediated activation of Src downstream of ERK/MAPK signaling. This particular PELP1 function has also been implicated in the development of tamoxifen resistance in transgenic mice [258]. HPIP, also known as pre-B cell leukemia homeobox interaction protein, is a microtubule-binding protein that is localized predominately in the cytoplasm. HPIP interacts with ERα through its C-terminus and promotes the activation of PI3K and Src. E2 has been demonstrated to induce the formation of an ERα/PI3K/Src/HPIP complex on microtubules that activates AKT/MAPK pathways in breast cancer cells [259]. MTA1s is a frameshift-derived shorter version of MTA1. MTA1s lacks a nuclear localization signal and it sequesters ERα in the

cytoplasm enhancing non-genomic signaling at the expense of the genomic functions. E2 signaling can also modulate the DNA damage response. E2 delays DNA repair by regulating ATR and Chk1 activation in breast cancer cells; interestingly, this is dependent on ERα mediated PI3K/AKT activation [260].

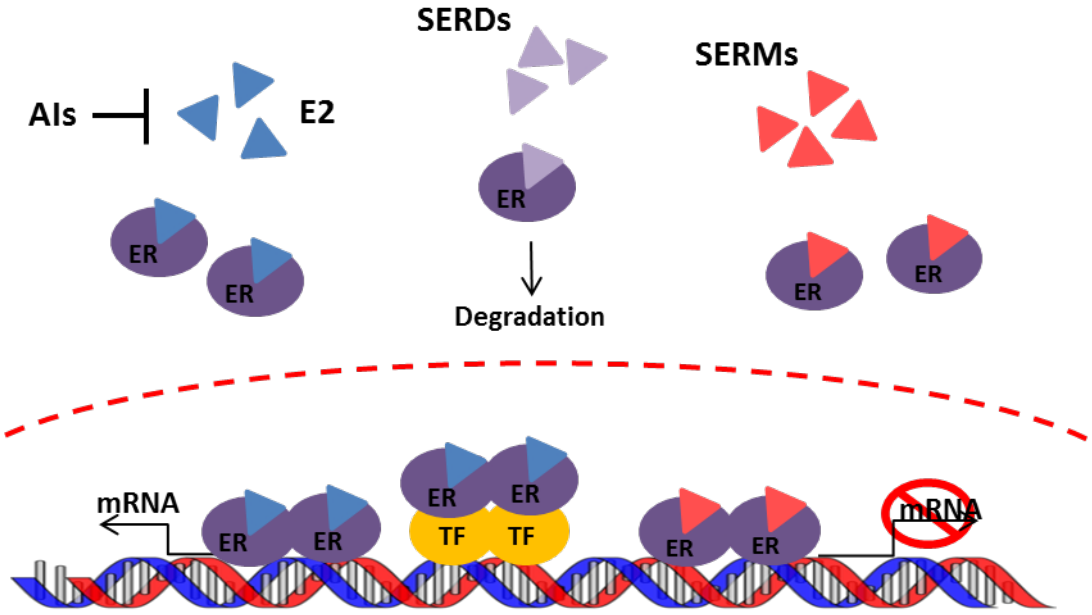

Figure 5. Targeting ER signaling in breast cancer. There are several classes of anti-estrogens used in the clinic to treat ER positive breast cancer. In the absence of anti-estrogens E2 binds to ER, inducing receptor dimerization and nuclear translocation. Activated ER can either interact with DNA directly or indirectly (with the aid of other DNA-binding transcription factors) to modulate gene transcription. Aromatase inhibitors (AIs) target the enzyme aromatase which is responsible for the production of E2. Selective estrogen receptor down regulators (SERDs) inhibit estrogen signaling by targeting the ER for degradation. Selective estrogen receptor modulators (SERMs) bind to ER resulting in a conformational change that can inhibit or promote the transcription of certain estrogen regulated genes depending on the cell context.

1.6 ENDOCRINE RESISTANCE IN BREAST CANCER

As previously discussed, the majority of breast cancers are ER+ and rely on E2 for their proliferation and progression, therefore, the most effective strategy to stop or slow the growth of hormone-sensitive tumors is to block E2 action using endocrine therapy. Current endocrine therapies for ER+ breast cancer include: tamoxifen, the selective ER modulator that competitively inhibits E2 binding to ERα; fulvestrant, the pure anti-estrogen that induces receptor degradation by the proteasome; and aromatase inhibitors (such as letrozole, anastrozole and exemestane) which target the enzyme responsible for estrogen production. For the past few decades, tamoxifen (TAM) has been the most widely used drug for the treatment of breast cancer with success both as a long-term adjuvant therapy and as a preventative agent [261-263]. Despite the fact that treatment with these anti-estrogens significantly decreases breast cancer mortality, a large number of patients fail to respond to initial therapy (*de novo* resistance) or develop resistance after prolonged treatment (acquired resistance). Anti-estrogen resistant (also known as endocrine resistant) breast cancers proliferate independent of E2 signaling. Several molecules and signaling pathways have been proposed to explain the development of endocrine resistance, often with significant overlap.

1.6.1 Growth factor signaling pathways

Perhaps one of the most extensively studied mechanisms regarding endocrine resistance is the upregulation of members of the epidermal growth factor family. Approximately one third of breast cancers overexpress human epidermal growth factor receptor 2 (HER2), and this is

associated with poor prognosis and survival. Tamoxifen induces the proliferation of the tamoxifen-resistant cell line MCF-7/HER2-18 (which overexpresses HER2), suggesting crosstalk between HER2 and ERα. Furthermore, epidermal growth factor receptor (EGFR or HER1) and insulin-like growth factor 1 receptor (IGF1R) are also known to be upregulated in endocrine resistance [264, 265]. Growth factor receptor activation results in increased signaling of downstream PI3K/AKT and MAPK/ERK kinase cascades, resulting in increased proliferation, survival, growth and motility. These pathways can provide alternative means to induce proliferation and survival to tumors when ER signaling is inhibited.

Overexpression of AKT has been demonstrated to confer resistance to tamoxifen and fulvestrant in breast cancer cells. Moreover, TAM resistant cells have higher levels of activated AKT [266]. Aside from providing an alternative proliferative pathway, activation of PI3K/AKT and MAPK/ERK kinase cascades contributes to endocrine resistance through posttranslational modifications of ERα and co-regulators (discussed below). As mentioned previously, ER can also engage in cytoplasmic and membrane signaling complexes which can activate various growth factor receptors. Interestingly, hyper-activation of these pathways increases non-nuclear ER localization and non-genomic activity, thus creating a positive feedback loop of cross-activation between the ER and growth factor receptor pathways. It is important to note that both estrogen and tamoxifen can activate non-genomic ER activity, thereby implicating tamoxifen in the development of its own resistance [267, 268].

1.6.2 Modifications of ER and co-regulators

One way to alter response to endocrine therapies is through modulation of ERα and its co-repressors and co-activators. Mutation or loss of expression of ERα has been reported in a subset of endocrine resistant breast cancers [269, 270]. Additionally, hyper-methylation of the ERα promoter region results in significantly reduced expression [271]. However, loss of ERα expression only accounts for a small portion of endocrine resistant breast cancers.

ERα phosphorylation has been associated with tamoxifen resistance. For example, phosphorylation of serine residues S118 (by MAPK), S167 and S305 (by AKT) can modulate the interaction of ERα with various transcriptional regulators and decrease sensitivity to tamoxifen *in vitro* [272, 273]. Thus far, only the S305 modification has been observed clinically in endocrine resistance [274, 275]. S305 phosphorylation alters the binding of ERα with the co-activator SRC-1, which prevents TAM induced inactivation of the transcription complex [276]. Phosphorylation of the methyl-transferase CARM1 by PKA allows the protein to bind to ERα independent of ligand, thereby preserving ERα transcriptional activity in the presence of tamoxifen. MED1, a subunit of the mediator co-activator complex that acts as a link between RNA polymerase II and transcription factors, is often overexpressed or phosphorylated in acquired tamoxifen resistance. Depletion of MED1 expression in cells restored sensitivity to tamoxifen, resulting in reduced proliferation [277]. Overexpression of the ER co-activator AIBI (amplified in breast cancer 1, also known as NCOA3 or SRC3) results in constitutive transcriptional activity of ERα, which leads to resistance to anti-estrogen treatment [278]. Additionally, downregulation of the co-repressor NCoR was reported in tamoxifen resistant

tumors. Finally, increased levels of ER transcription factors NFκB and AP-1 have also been associated with endocrine resistance [279, 280].

1.6.3 Alterations in cell cycle and apoptotic pathways

Another category of endocrine resistance mechanisms involves the modulation of proteins involved in cell cycle progression and induction of apoptosis. Tamoxifen and fulvestrant reduce cell proliferation by inducing G1 phase-specific growth arrest. Overexpression of proteins that induce the progression of the cell cycle such as c-Myc, cyclin E1, and cyclin D1, contribute to endocrine resistance [281, 282]. Cyclin D1 promotes G1/S phase transition and is over-expressed in 50% of breast cancers; overexpression of cyclin D1 in cell models results in tamoxifen resistance. C-Myc expression can contribute to resistance by decreasing the expression of the negative regulators of cell cycle progression p21 and p27. Moreover, reduced expression or stability of p21 and p27, as well as inactivation of the RB tumor suppressor, are also reported in tamoxifen resistance [283, 284]. It is important to note that multiple growth factor receptors and their downstream signaling pathways downregulate the expression or activity of cell cycle regulators. HER2 overexpression reduces p27 levels, and phosphorylation of p21 and p27 by Akt relocates the proteins to the cytoplasm and inhibits their activity.

Upregulation of anti-apoptotic molecules, such as Bcl2, decrease expression of pro-apoptotic molecules, such as BIK and caspase 9, can also lead to endocrine resistance [285]. As before, activation of growth factor receptor signaling via the PI3K/Akt pathway is important for the regulation of these apoptotic/survival molecules, but additional molecules that cross-talk with ERα, such as NFκB, have also been implicated [279].

1.6.4 Other mechanisms of endocrine resistance

Endocrine resistance can also be due to differences in drug metabolism and excretion. CYP2D6 is a member of the cytochrome P450 family which converts tamoxifen into its active metabolites 4-hydroxytamoxifen and endoxifen, which have 30-fold and 100-fold higher potency than tamoxifen respectively. Several polymorphisms of CYP2D6 alleles have been shown to reduce enzyme activity, resulting in a poor clinical outcome following tamoxifen treatment [286]. Additionally, overexpression of the drug efflux transporters such as P-glycoprotein/multi-drug resistance protein 1 (MDR1), might influence a patient's response to TAM by reducing overall drug and metabolite concentrations in the cell [287].

Another proposed mechanism in endocrine resistance involves the G-protein coupled receptor GPR30; a membrane bound receptor that mediates non-genomic estrogen signaling effects. Unexpectedly, both tamoxifen and fulvestrant bind to GPR30 and increase cell proliferation through the activation of EGFR-MAPK and cAMP signaling pathways, thereby enhancing estrogen signaling effects [288, 289].

Recent studies have implicated a role for non-coding RNA in the development of endocrine resistance, specifically microRNA (miRNA) and long non-coding RNA (lncRNA). miRNAs are small strands of RNA of 18-22 base pairs in length that regulate the expression of target mRNAs by inhibiting translation or degrading transcripts [290]. Expression profiles of miRNA in breast tumor samples have been associated with pathological features such as hormone receptor status and proliferation index, and miRNA gene regulation has been implicated in breast cancer progression [291, 292]. Several miRNAs are known to be involved in resistance and typically target genes implicated in cell cycle/apoptosis or in growth signaling

pathways. Overexpression of miR-221, miR-222 and miR-206 significantly reduce ERα expression in MCF-7 and T47D breast cancer cells, resulting in acquired tamoxifen resistance [293, 294]. Furthermore miR-221/222, along with miR-15a/16, have been shown to target genes involved in cell cycle inhibition (p27) and apoptosis (Bcl2) in HER2 positive breast cancers [295, 296]. Treatment of tamoxifen resistant MCF-7 cells with exogenous miR-15a/16 decreased tamoxifen-induced Bcl2 levels and re-sensitized the cells to tamoxifen toxicity [295]. Several miRNAs, including miR-519a, miR-301 and miR-101, all decrease PTEN expression and increase AKT signaling in endocrine resistant cells [297, 298]. Lastly, a recent study analyzing miRNA expression in matched samples from ER+ breast cancer patients treated with tamoxifen reported that high expression of miR-126 and miR-10a were associated with significantly longer relapse-free time after tamoxifen treatment [299].

Long non-coding RNAs (lncRNA) are RNA transcripts between 22-200 base pairs in length recently implicated in breast cancer gene regulation. The lncRNA BCAR4 was found to be overexpressed in tamoxifen resistant ZR-75-1 cells, and has recently been shown to correlate with increased invasiveness and tamoxifen resistance in human tumors [300].

1.7 NHERF1 IN BREAST CANCER

Recently, studies have implicated a role for NHERF1 in the regulation of cell proliferation in cancer. Overexpression of NHERF1 has been reported in breast cancer, schwannoma, and HCC

samples [301, 302]. Of the three, breast cancer has the most extensive analysis of the NHERF1 expression in cancer progression [302-306]. Nevertheless, conflicting evidence has made the role of NHERF1 in breast cancer enigmatic.

Interestingly, the NHERF1 gene contains several half-ERE sites in its promoter region and its transcription is regulated by estrogen in breast cells [162]. Furthermore, there is a significant correlation between NHERF1 and ER expression in human breast cancer [163]. Initial studies reported that overexpression of NHERF1 was significantly correlated with lower tumor invasiveness in breast cancer cell lines [305]. However, later studies analyzing a large number of breast tumor samples demonstrated the opposite, that NHERF1 overexpression is significantly associated with tumor stage, metastatic progression, and poor prognosis [302, 303].

Loss of one or both NHERF1 alleles has been reported in breast and ovarian cancers. One study reported a relatively low number (3%) of NHERF1 gene mutations in breast cancer cell lines and primary tumors associated with loss of heterozygosity (LOH), yet overall loss of at least one NHERF1(*SLC9A3R1)* allele was prevalent [305]. 58% of the breast cancer cell lines and 22% of the primary tumor screened had deletion of at least one NHERF1 allele. Two of the cell lines, MDA-MD-231 and SUM 149PT, harbored missense mutations (R180W and D301V) with LOH, and were subsequently shown to have reduced NHERF1 expression. The same study also reported a third mutation, K172N, which was found in DNA extracted from breast tumor tissue, but not in the adjacent normal tissue. Allelic loss of NHERF1 strongly correlated with tumor size and disease stage, which led the authors to postulate a tumor suppressor role for NHERF1 in breast cancer progression [305, 306].

NHERF1 interacts with several proteins implicated in the initiation and progression of cancer. One of the most studied is the interaction of NHERF1 with several members of the

PI3K/Akt pathway [307]. Loss of NHERF1 has been associated with increased Akt activation in MEF and breast cancer cells. NHERF1 has been shown to interact with epidermal-growth factor (EGFR) and platelet-derived growth factor receptors (PDGFR), as well as the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) [308, 309]. NHERF1 interacts with the C-terminus of EGFR and reduces its activation. PTEN antagonizes the activity of PI3K, and it has been demonstrated that NHERF1 increases PTEN stability and expression. One group reported a ternary complex between NHERF1, PDGFR, and PTEN, suggesting that NHERF1 may antagonize PDGFR signaling by promoting its association with PTEN [304, 310]. Furthermore, a recent study demonstrated that NHERF1 missense mutations identified in breast cancer tumors prevented the association of NHERF1 and PTEN. Taken together, these studies imply that NHERF1's role in cancer progression is at least in part due to its regulatory role in the PI3K/Akt pathway.

Our group recently reported that NHERF1 interacts with Fz receptors and antagonizes Wnt/β-catenin signaling in breast cancer cells [40]. Moreover, another group reported NHERF1 expression suppressed β-catenin signaling in mouse embryonic fibroblasts (MEF) cells. Interestingly, NHERF1 has also been shown to associate with β-catenin in HCC cells, where overexpression in the cytoplasm was observed to promote β-catenin transcriptional activity [26].

It has been postulated that differences in NHERF1 subcellular localization can explain the seeming contradicting roles of NHERF1 expression in cancer. The studies showing overexpression of NHERF1 in breast tumors report localization of the protein in the cytoplasm of cancer cells, as opposed to the typical membrane localization seen in normal mammary epithelial cells [163, 303, 311]. The same shift in NHERF1 subcellular localization has also been reported in HCC cells [26]. This has led to the hypothesis that NHERF1 acts as a tumor suppressor when

localized at the plasma membrane, but is an oncogenic protein when localized in the cytoplasm. One recent study has even reported nuclear expression of NHERF1 in a subset of breast tumors, and found that nuclear expression of NHERF1 was correlated with increased survival [304]. Considering the wide breadth of targets identified for NHERF1, the idea that NHERF1 function is highly dependent on its localization is an attractive one. However, more information is needed on how NHERF1 localization is regulated under pathological conditions.

1.8 RATIONALE AND AIMS

Our laboratory previously identified NHERF1 as a regulator of Wnt canonical signaling, demonstrating a direct interaction between NHERF1 and Fz receptors [40]. Initial studies suggested that NHERF1 may regulate Wnt/β-catenin signaling in mammary tissue. Preliminary data from the laboratory also suggested NHERF1 KO mice presented a form of communicating hydrocephalus characterized by pattern defects in the cilia of the ependyma, a phenotype potentially explained by a defect in Wnt PCP signaling. Interestingly, the Wnt PCP receptor Vangl2 also contains a class I PDZ ligand at the C-terminus. The observation that Vangl2 apical expression was lacking in NHERF1 KO ependyma led to the hypothesis that NHERF1 may regulate non-canonical as well as canonical Wnt signaling. Taken together, the evidence began to portray NHERF1 as a potential “signaling switch” between the Wnt pathways. Presumably, NHERF1 would regulate the signaling of these two pathways by inducing complexes that favor

the signaling of one pathway over another (turning on PCP signaling and turning off canonical signaling).

Our group sought to determine the role of NHERF1 in the regulation of both Wnt β-catenin signaling (specifically Wnt canonical signaling in breast cancer) and PCP signaling. This thesis is comprised of the following three specific aims: 1) Determining whether NHERF1 acts as tumor suppressor in a Wnt-driven breast cancer model, 2) Determining the role of NHERF1 expression and Wnt canonical signaling in endocrine resistance, and 3) Examining the role of NHERF1 in Wnt PCP signaling and ciliogenesis.

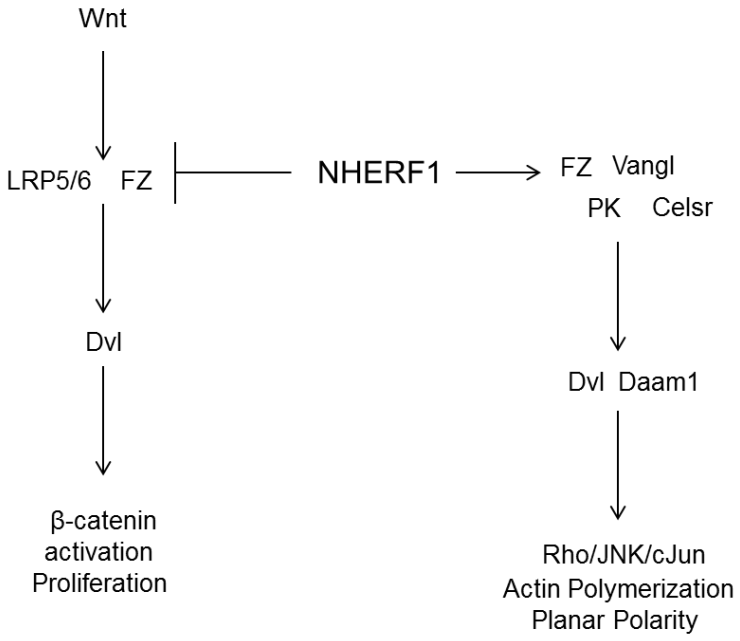

Figure 6. General model. We propose that NHERF1 acts as a molecular switch between Wnt/β-catenin and Wnt/PCP signaling; NHERF1 acts as a scaffold and increases the association of protein complexes that promote Wnt PCP signaling while simultaneously inhibiting the formation of complexes that promote Wnt/β-catenin activation.

2.0 NHERF1 AND CANONICAL WNT SIGNALING IN BREAST CANCER

Breast cancer is the most common cancer amongst women in the United States. Recently, several studies have implicated the PDZ scaffold NHERF1 in the development of breast cancer [301, 307]. NHERF1 overexpression decreases proliferation in several breast cancer cell models [306]. NHERF1 expression is upregulated by estrogen, a primary driver of breast cancer progression and its expression in normal mammary tissues is high. Similarly, inhibition of estrogen signaling by anti-estrogens such as tamoxifen and ICI 182,780 reduces NHERF1 expression [162, 163, 312]. Our laboratory recently identified NHERF1 as a novel suppressor of Wnt/β-catenin signaling in the mammary epithelium [40]. We hypothesized that NHERF1 may suppress Wnt canonical signaling and act as a tumor suppressor in a Wnt driven background. Furthermore, we our model predicted that loss of NHERF1 due to inhibition of estrogen signaling would increase activation of Wnt canonical signaling in the breast, potentially leading to resistance. In this study, we sought to determine whether NHERF1 could act as a tumor suppressor in a Wnt-driven breast cancer model. Furthermore, since NHERF1 is upregulated by estrogen, a crucial target in breast cancer therapy, we investigated the role of NHERF1 expression and Wnt canonical signaling in estrogen-independent and endocrine resistant breast cancer models.

2.1 INTRODUCTION

NHERF1, also known as Ezrin-binding protein 50 (EBP50), is a 50-kD scaffolding protein found abundantly in mammary epithelium. NHERF1 is characterized by two tandem N-terminal PDZ domains and a C-terminal Ezrin binding domain (EBD) that attaches NHERF1 to the cytoskeleton. NHERF1 interacts with several proteins including ion transporters, G-protein coupled receptors, receptor tyrosine kinases and other cellular components [24]. NHERF1 has recently been suggested to play a role as a tumor suppressor in breast cancer [302, 313, 314]. NHERF1 mutations occur in 3% of breast cancer cell lines and primary tumors, and loss of heterozygosity (LOH) at the NHERF1 locus in 50% of breast primary tumors [305]. Furthermore, NHERF1 mutations and LOH are associated with aggressive features of breast tumors, including tumor size, grade and stage. Knockdown of NHERF1 has been shown to increase cell proliferation in multiple breast cancer cell lines, while overexpression of NHERF1 was shown to induce apoptosis and inhibit cell proliferation [306]. Three naturally occurring NHERF1 breast cancer mutations have been reported: K172N, R180W, and D301V; these mutations are associated with larger tumor size, grade and more advanced disease state [305].

The majority of the studies that have investigated the mechanisms by which NHERF1 may regulate breast cancer progression have looked at NHERF1's role in PI3K/AKT signaling. NHERF1 interact with PTEN and increases it expression, thereby antagonizing PI3K activity and significantly reducing AKT activation. However, previous reports from our laboratory suggest that NHERF1 may also play a role in regulating another major proliferative pathway: the Wnt/β-catenin signaling pathway [40]. NHERF1 binds to Fz receptors, and its expression decreased β-catenin signaling in breast cancer cells [40]. More recently, others have reported increased

nuclear β-catenin accompanied by downregulated NHERF1 expression in polycystic kidney disease [315].

The oncogenic potential of Wnt/β-catenin signaling was first realized after the discovery that deregulation of the pathway by the mouse mammary tumor virus lead to mammary tissue transformation and hyper-proliferation. Mutations in Wnt signaling pathway components are rarely found in human breast cancer tumors, yet studies indicate that 60% of breast cancers have increased activity of β-catenin signaling [157, 159]. We have shown that NHERF1 regulates canonical Wnt signaling via direct interactions with a subset of Frizzled (Fz) receptors, the primary targets of Wnt, and that NHERF1 expression maintains low levels of β-catenin activity. Integrity of the NHERF1 PDZ2 domain is necessary for interaction with Fz receptors; two of the NHERF1 breast cancer mutations, K172N and R180W are located in the PDZ2 domain.

The role of NHERF1 expression in breast cells is further complicated by the fact that NHERF1 expression is upregulated by estrogen in breast cells. Most breast tumors express estrogen receptor (ER) and are driven by estrogen signaling [316]. Inhibiting ER signaling by either antagonizing the ER or depriving the tumor of estrogen (endocrine therapy) has been a focus of breast cancer treatment for many decades. There are three major classes of anti-estrogens used in the clinic: selective estrogen receptor modulators (SERMs), selective estrogen receptor down-regulators (SERDs), and aromatase inhibitors (AI) [317]. The most significant drawback of these therapies is the development of resistance. About 30% of the women receiving endocrine therapy develop recurrent tumors within 15 years [318]. The identification of specific signaling pathways activated in recurrent tumors is essential for the development of novel targeted therapeutic strategies.

Expression of NHERF1 in breast cancer cells is highly sensitive to changes in estrogen signaling. Treatment with estradiol increases NHERF1 mRNA and protein levels in estrogen responsive breast cancer cells. Conversely, SERMS that antagonize estrogen signaling decrease NHERF1 expression [162]. This, along with the observation that NHERF1 expression decreases β-catenin signaling in breast cancer cells in culture, leads to the hypothesis that loss of NHERF1 in breast cells following endocrine therapy could lead to increased Wnt/β-catenin signaling and acquired endocrine resistance.

To investigate the role of NHERF1 in Wnt signaling and breast cancer we proposed two specific aims: to determine whether NHERF1 acts as tumor suppressor in a Wnt-driven breast cancer model, and to examine role of NHERF1 expression and Wnt canonical signaling in endocrine resistance. We first attempted to transform the immortalized cell line MCF10A, which do not express NHERF1 endogenously, with ectopic expression of WNT1A and WNT3A ligands. Early experiments indicated that overexpression of Wnt ligands was not sufficient for transformation.

We then turned out attention to studying the role of NHERF1 expression and Wnt signaling in endocrine resistance. We tested the effects of the β-catenin inhibitor ICG-001 in estrogen-dependent and estrogen-independent breast cancer cell lines. Furthermore, we assessed the expression of NHERF1 and active β-catenin in primary and recurrent human breast tumors using immunohistochemistry (IHC) techniques. Results demonstrated that NHERF1 expression and endocrine resistance were not good predictors of ICG-001 sensitivity. Moreover, IHC results showed both positive and negative correlations between NHERF1 and active β-catenin expression. Overall, the results of these studies were inconclusive.

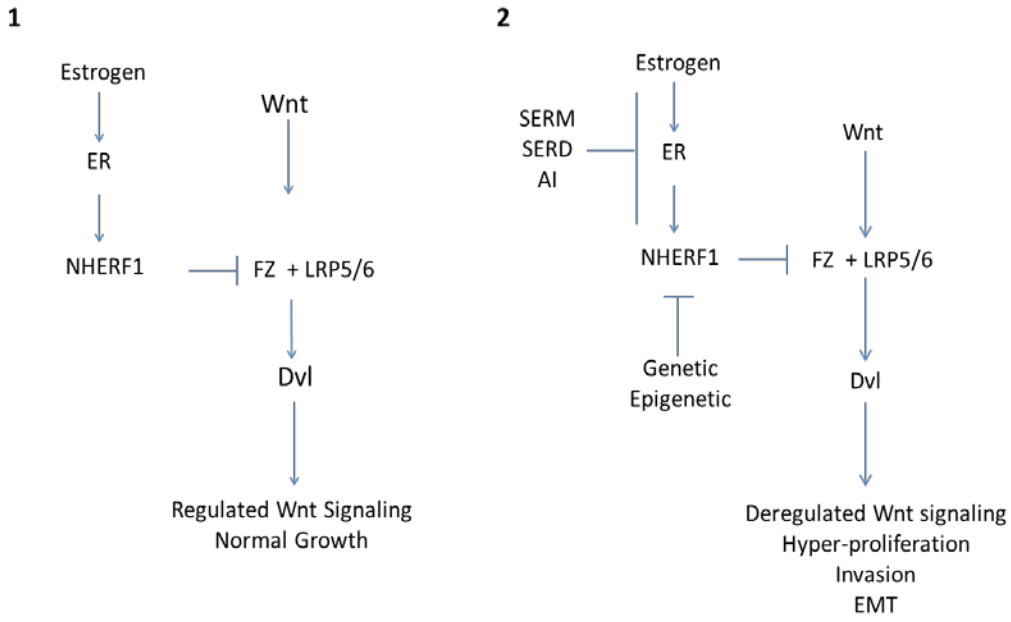

Figure 7. NHERF1's role in breast cancer. There are two main parts to our hypothesis: 1) In ER positive breast cancer, estrogen maintains relatively high levels of NHERF1 expression. In these cells, NHERF1 expression prevents Wnt induced proliferation and β-catenin activation. 2) In endocrine resistant breast cancer, NHERF1 function is abolished either through genetic mutation, epigenetic silencing, or inhibition of ER induced NHERF1 expression through pharmacologic intervention (SERMs, SERDs, and A.Is). This leads to deregulated Wnt signaling and cancer progression

2.2 MATERIALS AND METHODS

Reagents: All reagents were purchased from Sigma unless otherwise noted.

Cell culture and generation of cell lines: All cell lines were cultured at 37 degrees C with 5% CO2. MCF10A cells were purchased from ATCC and cultured in MEBM supplemented with 10% horse serum, 1% Pen/Strep and 100 ng/mL cholera toxin. Stable Wnt producing cell lines were generated by transfecting with Wnt1a and Wnt3a plasmids and selecting with increasing concentrations of the mammalian selection agent G418 (100-400 μg/mL) over the course of

several months. Two different MCF-7 parental cell lines were used during the course of this study, one low passage cell line from ATCC (under 50 passages) and one high passage (over 150 passages). Both MCF-7 parental cell lines were cultured with DMEM high glucose media supplemented with 10% fetal bovine serum (FBS) and 1% Pen/Strep. T47Ds, another cell culture model for luminal breast cancer, was also used and cultured in RPMI high glucose media supplemented with 10% FBS and 1% Pen/strep. We used 3 different estrogen-independent cell lines derived from MCF-7s: MCF-7 LY2s, MCF-7 TamR and MCF-7 LTED, and two estrogen-independent cell lines derived from T47Ds named T47D LTED and T47D TamR. All T47D cell lines were provided by the Dr. Oesterreich laboratory at the Magee's Women's Research Institute. MCF-7 LTEDs were graciously provided by the laboratory of Dr. Richard Stanten at the University of Virginia, School of Medicine. MCF-7 TamR cells were sourced from the laboratory of Dr. Guoyong Wang at Tulane University School of Medicine. All estrogen-independent cell lines were cultured in phenol red free DMEM supplemented with 10% charcoal stripped FBS and 1% Pen/Strep (herby referred to as E2-free media). 300 nM 4-hydroxy-tamoxifen was added to the media of all TamR and LY2 cell lines to maintain SERM resistance phenotype.

Adhesion-dependent growth assays: MCF10A control and MCF10A Wnt3a and Wnt1a stably transfected cells were seeded onto 12 well plates at a density of 20,000 cells/well. Total cell number per well was assessed using an Invitrogen automated Cell Countess at days 2, 4 and 6 post plating.

Soft agar colony formation assays: Anchorage independent growth of MCF10A ctrl, WNT1A and WNT3A cell lines was tested using soft agar colony assays in 6 well plates. 2X MCF10A cell culture media and agarose were mixed under sterile conditions to final

concentrations of 0.7% agarose for the base layer, and top layer of 0.35% agarose. Cells were seeded at a density of 30,000 cells/well and fed with fresh medium twice a week. Parallel experiments with MCF-7 cells were used as a positive control. After 4 weeks, wells were stained for one hour at room temperature in a solution of 0.01% crystal violet and 10% ethanol to detect colonies.

NHERF1 Expression in SERM treated cells: MCF-7 parental (high passage) cells were plated on 10 cm plates and rinsed a minimum of 3 times over 24 hours with E2-free media to remove excess estrogen. After 24 hours, cell were seeded onto 6 well plates and treated with E2-free media supplemented with 1 nM E2. Once cells attached, they were treated with either vehicle control, 5-15 µM Raloxifene or 5-15 µM toremifene. After 72 hours, cells were harvested using 300 microliters of RIPA lysis buffer (50 mM Tris-Hcl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) per well and incubated on ice for 20 minutes. Lysate protein concentration was determined using Pierce BCA protein assay. Samples were diluted 1:1 with 2X laemli sample buffer and incubated at 100°C for 5 minutes. 30ug of lysate were loaded onto gels and transferred onto PVDF membranes. Blots were blocked for 1 hour at room temperature using 5% skim milk in PBST (Phosphate buffered Saline with 0.1% Tween). After blocking, blots were incubated overnight in blocking buffer supplemented with one of the following primary antibodies at 4°C: 1:500 NHERF1 (Santa Cruz), 1:300 cyclin D1 (Santa Cruz), and 1:10000 α-tubulin (Abcam).

BrdU proliferation assays: MCF-7 parental (high passage) cells were seeded onto 25 mM coverslips at low density in E2 free media with either vehicle control (0.1% Ethanol) or 1 nM E2. Cells cultured without estrogen were rinsed 2-4 times daily for 48 hours. After 48 hours, cells were treated with fresh media with a final concentration of 50% of either L cell

control (a murine fibroblast cell line) or Wnt3a conditioned medium (from an L cell line that constitutively expresses and secretes Wnt3a), with either 1 nM E2 or vehicle control. 24 hours after treatment with L Cell or Wnt3a medium, 0.03 mg/mL BrdU was added and cells were incubated for another 2 hours at 37°C. After BrdU incorporation, coverslips were fixed with cold 70% Ethanol for 5 minutes and subsequently rinsed thoroughly with PBS. Coverslips were incubated in 1.5 M HCl for 30 minutes at room temperature and then rinsed with PBS before blocking for one hour with blocking buffer (1% BSA and 5% goat serum in PBS). After the cells had been blocked, 100 µl of 1:1000 BrdU antibody (Millipore) in blocking buffer was added to each coverslip and incubated at 4°C overnight in a humidified chamber. Following incubation with primary antibody, coverslips were rinsed with PBS and treated with 1:1000 goat anti-mouse Alexa Fluor 546 (Life Technologies) in blocking buffer for one hour at room temperature. Coverslips were rinsed with PBS and incubated with 1ug/mL DAPI (Roche diagnostics) in PBS for 10-20 seconds, rinsed thoroughly with PBS and mounted onto slides using Flurogel mounting media. For analysis, images of a minimum of 10 random fields were collected using confocal microscopy and percent BrdU positive cells were calculated using Image J.

Wnt Rescue of SERM toxicity: High passage MCF-7 parental cells were cultured in E2 free media for 24 hours and rinsed 3-5 times to remove excess estrogen. After 24 hours, cell were plated on 96 well plates at 3,000 cells per well in E free media (plated at 100 µl per well). Cells were allowed to attach for 16-24 hours and 100 µl of either L cell control or L cell Wnt3a conditioned media containing 2X concentrations of E2 and various concentrations of raloxifene or toremifene was added to each well for a total of 8 different SERM concentrations including vehicle control (1:1000 DMSO). Final concentration was 100 pM E2 for all wells and 100 nM-

30 µM of either raloxifene or toremifene with a minimum of 4 replicates per concentration. Total DNA was measured 4 days post treatment with SERMS using a CyQuant assay system (ThermoFisher Scientific C35006). After 4 days, all media was removed and cells were incubated at 37°C for one hour with 100 µl/well of CyQuant DNA dye. Fluorescence was measured using a Wallace Victor plate reader under manufacture's Fluorescein settings (485 ex/535 emission for 0.1 seconds).

ICG-001 proliferation assays: ICG-001 was purchased from Tocris Bioscience (Cat. No.4505). For ICG-001 proliferation assays, cells were plated on 96 well plates at approximately 3000 cells/well (in 100 µl). 24 hours after plating, cells were treated with vehicle control (0.1% DMSO) or ICG-001 to a final concentration ranging from 30 nM- 30 µM. Total DNA was measured using the CyQuant assay (as mentioned above) 4 days post treatment with ICG-001. In these experiments, parental cells were plated in regular growth medium regular estrogen-containing FBS, and in some experiments as an extra control media was supplemented with 10 nM E2 for all wells to test ICG-001 toxicity in the presence of excess estrogen. All estrogen-independent cell lines were plated in E2 free media with 300 nM 4-hydroxy-tamoxifen when appropriate.

*TOP-Flash only assays***:** TOP-Flash luciferase reporter plasmids were purchased from Addgene. Cells were seeded on 6 well plates in their respective media and transfected with 2ug/well of TOP-Flash luciferase reporter constructs using Fugene 6 transfection reagent at a 3:1 ratio according to manufacture instructions. 8 hours post-transfection, 5-20 µM ICG-001 or vehicle control was added to well and cells were incubated at 37°C for 24 hours. After 24 hours, cells were lysed with Promega Passive Lysis Buffer and incubated at room temperature for 15 minutes. Protein concentration was determined using Pierce BCA protein assay. Luciferase

activity was measured by adding 100 µl of Promega Luciferase Assay Reagent (LAR) to 20 µl of each sample and reading immediately on a manual luminometer. Total luminescence was normalized to protein concentration for each sample.

TOP-Flash and Renilla Luciferase assays: Renilla luciferase plasmids were graciously provided by the laboratory of Dr. Monga at the University of Pittsburgh, Department of Pathology. For TOP-Renilla luciferase assays, cells were seeded onto 6 cm plates and transfected with 5 µg of DNA per plate (3 µg of TOP-Flash reporter and 2 µg of Renilla reporter plasmids) using Xtreme-gene HP transfection reagent (Roche) at a 3:1 ratio according to manufacture instructions. Cells were incubated at 37°C for 24 hours post transfection and then lysed with 300 µl of Promega Passive Lysis Buffer per plate and scraped off of plates using a plastic spatula and sonicated for 5 minutes (10 second pulses at 4°C). TOP-Flash luciferase activity was measured by adding 100 µL of Promega Luciferase Assay Reagent to 20 µl of cell lysate. Immediately after TOP-Flash reading was collected, 100 µl of Promega Stop&Glo buffer was added to each sample to stop TOP-Flash reporter activity and initiate Renilla luciferase activity. Both measurements were done on a Wallace Victor plate reader.

TUNEL assays: MCF-7 parental and estrogen-independent cells were seeded onto poly-D-lysine coated 22 mm coverslips in 6 well plates and allowed 16-24 hours to attach. Cells were treated with 20 µM ICG-001 or vehicle control for 48 hours. For a positive control, parental MCF-7s were treated with 20 µM raloxifene in parallel. After incubation with ICG-001 or raloxifene, cells were fixed with 4% paraformaldehyde on ice for 20 minutes and then rinsed three times with PBS. Cells were then permeabilized with 0.2% Triton X-100 PBS solution and rinsed with PBS. The coverslips were covered with 100 µl of Promega TUNEL Equilibration buffer and incubated at room temperature for 15 minutes. After equilibration, coverslips were

covered with 50 µl of rTdT buffer and incubated at 37°C for 1 hour in a humidified chamber. To terminate the nucleotide incorporation reaction, coverslips were incubated in 2X SCC buffer for 15 minutes at room temperature. Afterwards, coverslips were rinsed with PBS and nuclei were counterstained by immersing the slides in 1 µg/mL DAPI PBS solution for 10-30 seconds. Cells were then rinsed three times with PBS; coverslips were mounted onto glass microscope slides using Flurogel mounting media. Samples were analyzed on an Olympus Fluoview confocal microscope.

Immunohistochemistry: Paraffin embedded human breast tissue samples from primary and recurrent tumors were acquired from the Health Sciences Tissue Bank at the University of Pittsburgh. Tissue sections were de-paraffinized by incubating slides in fresh xylene twice for 10 minutes each time. Slides were washed twice with 100% ethanol for 3 minutes. Samples were then rehydrated by sequentially immersing slides through graded ethanol washes (100%, 95%, 70%, and 50%) for 3 minutes each. Following rehydration, slides were rinsed with deionized water and then PBS for 5 minutes. To perform antigen retrieval, samples were placed in boiling citrate buffer (10 mM citric acid, 0.05% Tween, pH 6.0) and incubated in an 80°C water bath for one hour. Tissue samples were then blocked with blocking buffer (5% goat serum and 1% BSA in PBS) for 1 hour at room temperature. After blocking, samples were covered with blocking buffer containing 1:150 NHERF1 (Pierce antibodies) and 1:300 Active β-catenin (Millipore) antibodies and incubated at 4° C overnight in a humidified chamber. Slides were then thoroughly rinsed with PBS and incubated at room temperature for 1 hour in blocking buffer containing 1:1000 dilutions of both goat anti-rabbit Alexa fluor 555 (Life Technologies) and goat anti-mouse Alexa fluor 488 (Life Technologies). Samples were then incubated in 1µg/mL DAPI (Roche) for 15 minutes and then thoroughly rinsed with PBS before mounting with Flurogel

mounting media. Slides were imaged on an Olympus Fluoview confocal microscope. To determine relative protein expression, laser and microscope settings were kept the same for each matched pair set and relative fluorescence was quantified using Image J software.

β-Catenin Western blots: For detection of **β**-catenin**,** cell lysates from TOP-Renilla assays (collected in Promega passive lysis buffer) were used. 20 µg of cell lysates were loaded onto Tris-HCl 7.5% gels (BioRad) and separated using SDS-PAGE electrophoresis. Gels were transferred onto a PVDF membrane and blocked for 1 hour at room temperature in blocking buffer (5% BSA in TBST). Blots were then incubated at 4°C overnight in blocking buffer containing one of the following primary antibodies: 1:3000 Total β-catenin (Millipore), 1:1000 S675 phospho-β-catenin (Cell signaling), 1:1000 S552 β-catenin (Cells signaling), 1:1000 Y654 β-catenin (Abcam), 1:1500 Active β-catenin (Millipore), and 1:10000 α-tubulin (Abcam). After incubation with primary antibody, blots were rinsed with TBST (3 times, ten minutes each rinse) and incubated for 1 hour at room temperature in blocking buffer with either 1:5000 goat anti-mouse or goat anti-rabbit HRP conjugated secondary antibodies (BioRad and #1721011 and #1706515 respectively). Blots were developed using Pierce ECL substrate according to manufacturer's protocol. Relative protein expression was calculated using Image J software.

Table 1. Antibodies used in chapter 2

Antibody	Type	Species	Source
active-β-catenin	primary	Mouse	Millipore 05-665
anti-rabbit Alexa 555	secondary	Goat	Life Technologies A-21428
anti-mouse Alexa 488	secondary	Goat	Life Technologies A-11001
anti-mouse Alexa 546	secondary	Goat	Life Technologies A-11030
anti-mouse HRP	secondary	Goat	BioRad 1721011
anti-rabbit HRP	secondary	Goat	BioRad 1706515
alpha-tubulin	primary	Rabbit	Abcam ab4074
β-catenin	primary	Rabbit	Millipore 06-734
BrdU	primary	Mouse	Millipore 05-633
cyclin D1	primary	Mouse	Santa Cruz DCS-6 sc-20044
NHERF1	primary	Rabbit	Santa Cruz H-100 sc-134485
NHERF1	primary	Rabbit	Pierce antibodies PA5-17044
S552 β-catenin	primary	Rabbit	Cell Signaling 9566
S675 β-catenin	primary	Rabbit	Cell signaling 4176
Y654 β-catenin	primary	Rabbit	Abcam ab59430
Wnt-1	primary	Rabbit	Santa Cruz H-89 sc-5630
Wnt-3a	primary	Mouse	Santa Cruz 3A6 sc-136163

Table 2. Plasmids used in chapter 2

Plasmid	Vector	Source
TOP-Flash	pTA-Luc	Addgene
Renilla-Flash	pTA-Luc	Provided by S. Monga laboratory
Wnt-1	pcDNA3	Addgene
Wnt-3	pcDNA3	Addgene

2.3 RESULTS

2.3.1 Ectopic expression of Wnt ligands in MCF10As does not induce transformation.

To determine if NHERF1 could act as a tumor suppressor in a Wnt driven background, we first wanted to see if ectopic expression of Wnt ligands could transform a breast cancer cell line that did not express NHERF1 endogenously. For this purpose, we chose MCF10A cells, a non-tumorigenic mammary epithelial cell line often used in oncogenesis experiments that does not express NHERF1 [319]. We created two Wnt ligand producing stable cell lines derived thereof: MCF10A Wnt1a and MCF10A Wnt3a.

Preliminary data suggested that ectopic expression of Wnt ligands did not significantly increase in anchorage dependent proliferation. However, MCF10A Wnt3a and MCF10A Wnt1A cells failed to form colonies in soft agar assays (data not shown), a result confirmed by reports from independent studies [320].

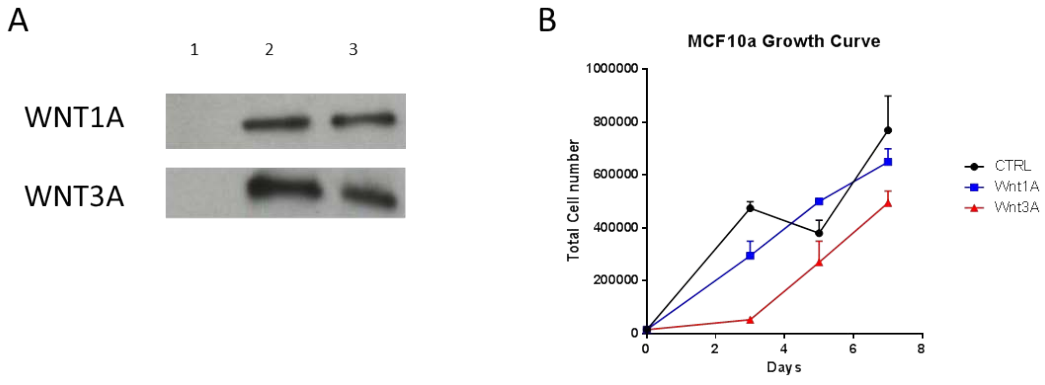

Figure 8. Wnt ligand overexpression in MCF10As. A) MCF10A were transfected with WNT1A and WNT3A genes and selected with increasing concentrations of G418 (final concentration 400 µg/mL) over several weeks.

Colonies were selected and expanded before testing for Wnt ligand expression using Western blotting techniques. Lane 1: MCF10As transfected with empty vector. Lanes 2&3: Cells derived from colonies transfected with Wnt ligand. B) Ectopic expression of Wnt ligands does not significantly increase the proliferation of MCF10A cells. MCF10A, MCF10A Wnt1a and MCF10A WNt3a cells were seeded onto 12 well plates at 20,000 cells/well and cell number was assessed at 2, 4 and 6 days post plating.

2.3.2 Wnt3a increases proliferation in estrogen deprived MCF-7 cells

The proposed model predicts that breast cancer cells would only be sensitive to the proliferative effects of Wnt ligands in the absence of estrogen. To model ER+ breast cancer, we chose MCF-7 cells, an ER+ luminal breast cancer cell line that is highly sensitive to the proliferative effects of estrogen. To test proliferation, we measured BrdU (5-bromo-2'-deoxyuridine) incorporation in MCF-7 cells following treatment with Wnt3a or L cell conditioned media in the presence and absence of estrogen. BrdU is a thymidine nucleotide analog that is incorporated into the nuclear DNA of cells in S-phase, and thus can be used to identify actively dividing cells. As demonstrated in Figure 9, in the presence of 1 nM E2, MCF-7s have a high number of BrdU positive cells which is not significantly changed by the presence of Wnt3a ligands. However, in the absence of estrogen basal proliferation decreases greatly, and addition of Wnt3a conditioned media significantly increases BrdU+ cells from 18% to 40% ($p<0.01$). This suggests that in estrogen responsive cells, estrogen is the primary driver of proliferation and the presence of Wnt ligands is redundant. However, when estrogen is absent, Wnt is able to significantly increase the proliferation of estrogen responsive cells.

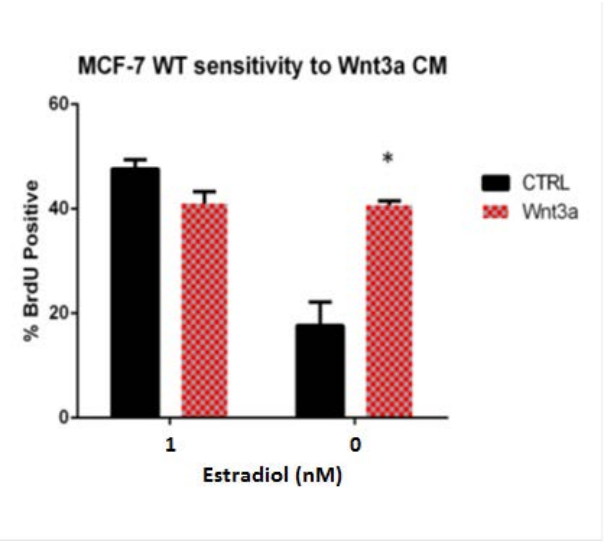

Figure 9. Parental MCF-7s only respond to Wnt in the absence of estrogen. MCF-7s were incubated in either control (L cell media) or Wnt3a conditioned media for 24 hours and cell proliferation was measured using BrdU nucleotide incorporation assay. In the presence of estradiol, Wnt3a does not significantly increase BrdU positive cells. However, following acute estrogen deprivation, Wnt3a is able to significantly increase the number or proliferative cells. N=3. *p<0.01

2.3.3 Wnt3a rescues cells from raloxifene but not toremifene toxicity

Previous studies have demonstrated that NHERF1 expression is regulated by estrogen in breast cancer cells. Tamoxifen and fulvestrant have both been demonstrated to decrease NHERF1 expression [315]. If our hypothesis that loss of NHERF1 contributes to a Wnt-driven endocrine resistant phenotype is correct, then identifying a SERM which does not decrease NHERF1 expression could prove to be clinically relevant. To this end, I tested the effects of treatment with raloxifene and toremifene on NHERF1 expression in parental MCF-7s. Western blot analysis revealed a significant decrease in NHERF1 expression after 72 hours in cells treated with raloxifene, but not toremifene (Fig. 10A-B). The expression of cyclin D1, a protein whose expression is heavily regulated by estrogen in MCF-7 cells, was used as a control for estrogen signaling inhibition (Fig. 10C). Interestingly, high concentrations of toremifene have been

clinically shown to be effective towards a subset of tamoxifen resistant patients, suggesting that differences between these SERMs may be relevant for acquired endocrine resistance [321].

If loss of NHERF1 expression in breast cancer cells is indeed responsible for increased sensitivity to Wnt ligands, then one would expect Wnt ligands to rescue cells from raloxifene but not toremifene toxicity. To test this hypothesis, MCF-7 parental cells were treated with various concentrations of either raloxifene or toremifene in the presence and absence of Wnt3a ligands. Results show that while Wnt3a conditioned media significantly increases the IC50 of raloxifene (4.7 μM vs 17 μM), it does not significantly change the toxicity of toremifene (Fig. 10 D-E).

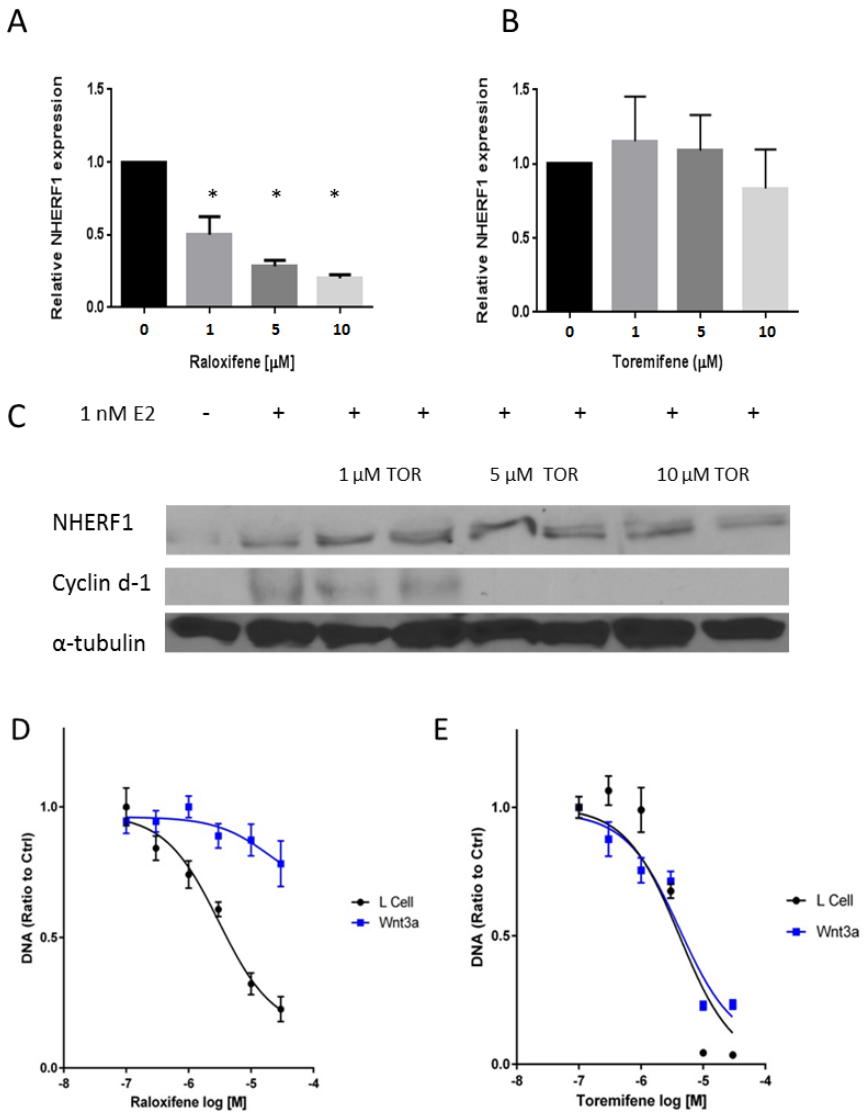

Figure 10. Wnt3a rescues cells from raloxifene but not toremifene toxicity: TOP: MCF-7 parental cells were treated with either vehicle control, raloxifene (1-10 μM) or toremifene (1-10 μM) in media supplemented with 1 nM E2. After 72 hours, NHERF1, alpha-tubulin and cyclin D1 expression were determined using Western blotting techniques and quantified using Image J. All data represents a minimum n of 3. **A)** Quantification of NHERF1 shows a significant and concentration dependent decrease in raloxifene treated cells. **B)** Toremifene does not significantly decrease NHERF1 expression after 72 hours. **C)** Representative Western blot demonstrating reduction of estrogen-induced cyclin D1 expression, but not NHERF1 expression, in toremifene treated cells. *$p<0.01$ ANOVA statistical analysis. **BOTTOM:** MCF-7 parental cells were seeded onto 96 well plates at treated with vehicle ctrl, or 0.1-30 μM of SERMs in the presence of 100 pM E2. Cells were also supplemented with either 50% L cell (control) or Wnt3a conditioned media. After 4 days, total DNA was measured using CyQuant proliferation assays. Results indicated that Wnt3a rescued cells from raloxifene growth arrest **(D)**, but not toremifene **(E)**. Results represent an n of three for each experiment.

2.3.4 ICG-001 inhibits the proliferation in several breast cancer cell lines

To test the hypothesis that NHERF1 expression and Wnt canonical signaling play a role in the development of endocrine resistance, we investigated the effects of Wnt/β-catenin signaling inhibition in endocrine sensitive and endocrine resistant breast cancer cell lines. To model estrogen driven breast cancer, we used the MCF-7 cells mentioned previously (referred to as MCF-7 parental cells). Three separate estrogen-independent cell lines derived thereof were used to model endocrine resistant breast cancer: MCF-7 long term estrogen deprived (LTEDs), MCF-7 tamoxifen resistant (TamR) and MCF-7 LY2s. MCF-7 LTED, as the name would suggest, are an MCF-7 derived cell line that had been cultured in estrogen free media for over a year. MCF-7 TamRs were derived by culturing cells in increasing dosages of 4-hydroxy-tamoxifen (up to 100 nM), while MCF-7 LY2s were originally cultured with increasing concentrations of the raloxifene analog LY 117018 (up to 1 μM).

To determine whether Wnt/ β-catenin signaling was necessary for the proliferation and survival of MCF-7 parental and derived cell lines, cells were seeded onto 96 well plates and treated with either vehicle control or various concentrations of ICG-001 (0.1-30 μM). ICG-001 inhibits the association of CBP and β-catenin in the nucleus, thus preventing β-catenin mediated transcription. Four days after treatment with ICG-001, total DNA was measured using a CyQuant proliferation assay. Results showed that ICG-001 significantly inhibited the proliferation of MCF-7 LTED, TamRs and LY2 cells, but not the proliferation of the parental cell line (Fig. 11A). Additionally, ICG-001 inhibits the TOP-flash reporter activity of the estrogen-independent cell lines but not the parental (high passage) MCF-7s (Fig. 11B). These

results suggest that β-catenin signaling may play a major role in the proliferation of breast cancer cells in the absence of estrogen signaling.

We speculated that inhibition of β-catenin signaling with ICG-001 could potentially increases apoptosis in the estrogen-independent cell lines. TUNEL assay techniques were used to measure apoptosis in MCF-7 TamR and LTED cells treated with ICG-001 or vehicle control. Results indicated that ICG-001 did not significantly increase the number of apoptotic cells after 48 hours. Raloxifene treatment of parental MCF-7s was used as positive control for apoptosis.

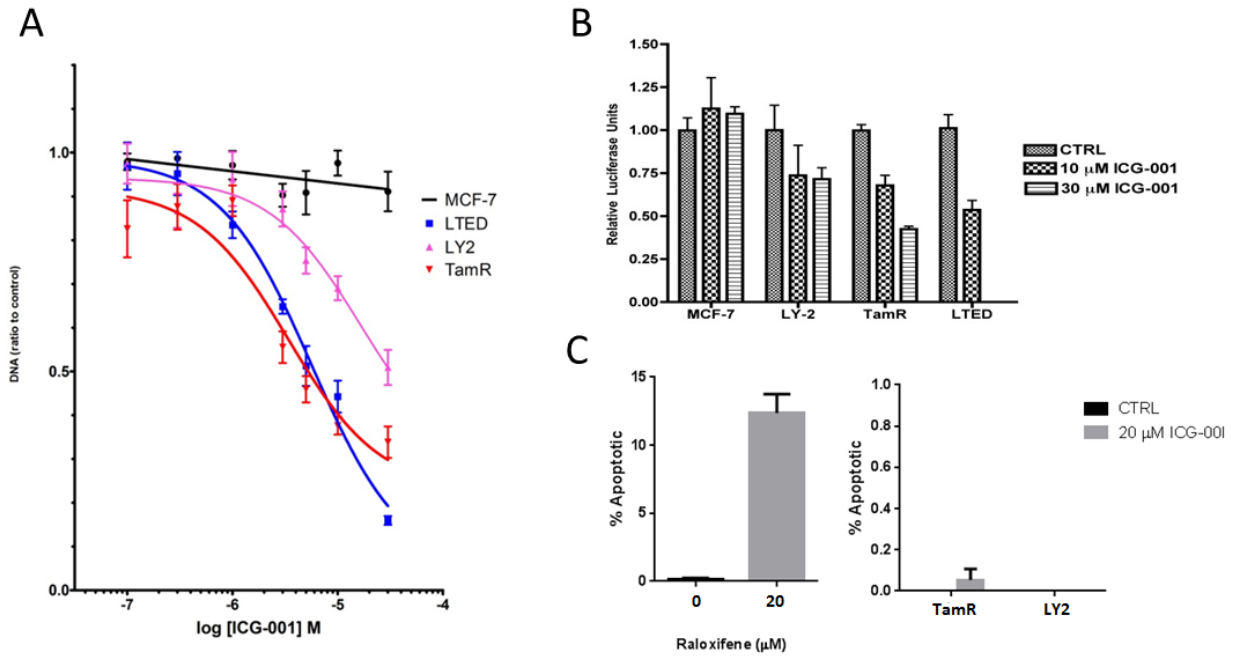

Figure 11. ICG-001 inhibits the proliferation of several endocrine resistant cell lines. A) MCF-7 LTEDs, TamRs, LY2 and parental cell lines were treated with either vehicle control or 0.1-30 μM of ICG-001, after 4 days total DNA was measured using a CyQuant proliferation assay. All samples were normalized to vehicle control. **B)** MCF-7 parental and derived cell lines were transiently transfected with TOP-Flash luciferase constructs and treated with either vehicle control, 10 or 30 μM ICG-001for 24 hours. After 24 hours, luciferase activity was measured and normalized to total protein. Results show that acute treatment with ICG-001 causes a significant decrease in TOP-Flash reporter activity in the MCF-7 LY2, TamR and LTED cell lines, but not in the parental estrogen-dependent MCF-7s. **C)** Cells were seeded onto poly-D-lysine coverslips and apoptosis was assessed using TUNEL assay techniques. LEFT: As a positive control for apoptosis, MCF-7 parental cells were treated with either vehicle control or 20μM raloxifene for 48 hours. TUNEL results indicate a robust increase in apoptosis from 0.12% to 12.4%.

RIGHT: MCF-7 TamR and LTEDs were treated with vehicle control of 20μM ICG-001 for 48 hours. In these samples virtually no apoptotic cells were detected (0% in control vs to 0.1% for ICG-001 treated cells).

2.3.5 NHERF1 expression in endocrine resistance

Unexpectedly, Western blots showed that NHERF1 expression in the estrogen-independent MCF-7 derived cell lines is comparable to parental cells in the presence of 1 nM E2 (Figure 12 A). Acute deprivation of estrogen in the parental MCF-7s, however, reduced NHERF1 expression by about 45%, consistent with the reports published from other groups (Fig 12 B).

Although NHERF1 expression remained high in the estrogen-independent cell lines, we speculated that NHERF1 expression could convey an advantage to cell culture conditions that may not reflect the effects of chronic estrogen deprivation *in vivo*. Therefore, we determined the expression of NHERF1 and activated β-catenin in human breast cancer tissue. Paraffin embedded human breast cancer samples of primary and recurrent breast tumors (after treatment with tamoxifen) provided by UPCI were probed for NHERF1 and hypo-phosphorylated "activated β-catenin", or β-catenin that is un-phosphorylated at sites S33, S37 and T41 (the phosphorylation sites for the CK1 and GSK3 kinases) using immunohistochemistry (IHC) techniques as described above. Human breast IHC results show great variation in changes of NHERF1 and β-catenin expression between different matched pairs (Fig. 12C). While the sample size is inherently limiting (n=6), the data so far collected are inconclusive about the correlation between NHERF1 signaling and activated β-catenin in primary and recurrent tumors. On the one hand, two of the six samples (B and C) have increased active β-catenin and decreased NHERF1 expression in the recurring tumor as predicted by our model. One matched pair showed an unexpected increase in NHERF1 expression accompanied by decreased β-catenin activation (Fig. 12C, pair D), while another showed a significant decrease in β-catenin activation and a small

decrease in NHERF1 expression (Fig. 12C, pair F). However, other samples show very little change in expression of either protein between tumor pairs (Fig. 12C).

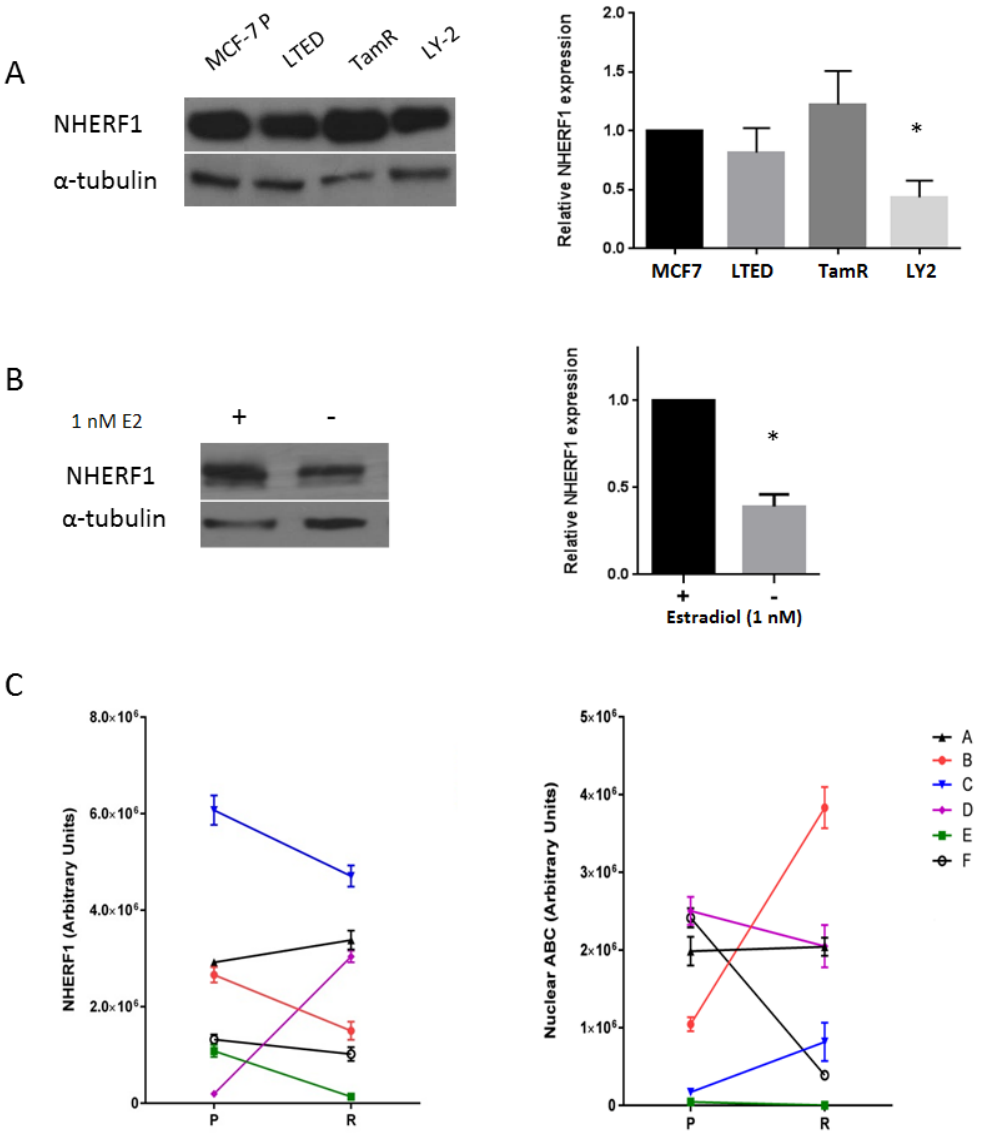

Figure 12. NHERF1 expression in endocrine resistance: A) LEFT: Representative western blot of NHERF1 expression in MCF-7 parental, LTED, TamR and LY-2 cell lines. RIGHT: Protein expression was quantified using Image J software and normalized to α-tubulin loading control. Results show that MCF-7 LY2s are surprisingly the only cell line with significantly reduced NHERF1 expression (as determined by ANOVA statistical analysis), n=4 *p<0.01. **B)** LEFT: Representative Western blot of NHERF1 expression in MCF-7 parental cells acutely deprived of

estrogen and in cells supplemented with 1 nM E2. **C)** Results of IHC staining of human primary (P) and recurrent (R) breast tumors. LEFT: Quantification of NHERF1. RIGHT: Quantification of "activated" β-catenin in the nucleus.

2.3.6 The Effects of ICG-001 in other cell culture models

To determine if the differences in ICG-001 sensitivity between parental and estrogen-independent cell lines were a generalized phenomenon, we extended the ICG-001 studies to T47-Ds, a luminal breast cancer cell line, and estrogen-independent cell lines derived thereof: T47D LTED and T47D TamR. Unexpectedly, ICG-001 inhibited the proliferation of T47D LTED, TamR and parental cell lines, despite the presence of estrogen in the parental cell lines (Fig. 13A). The IC50 of the parental T47Ds in the estrogen containing media, 3.4 μM, is similar to the IC50 of β-catenin inhibition reported in colon cancer cell lines (3 μM) [322]. These results suggested that the sensitivity to ICG-001 in breast cancer cell lines could be an effect that is independent of the presence of estrogen signaling. We tested this new hypothesis by extending the ICG-001 proliferation experiments to a different set of estrogen-dependent MCF-7s. These new MCF-7s were low passage MCF-7 cells (MCF-7 ATCC). Results showed that ICG-001 does inhibit the growth of MCF-7 ATCC (IC50: 4.9 μM), despite the presence of estrogen in the regular non-charcoal stripped media (Fig. 13 B). Addition of excess E2 (10nM) to the media did increase the IC50 to approximately 17 μM, however this is similar to the IC50 of the MCF-7 LY2s, which were culture in media without estrogen and 100 nM 4-hydroxy-tamoxifen. Paradoxically, preliminary data from TOP-Renilla assays show higher β-catenin signaling in the endocrine resistant cell lines than MCF-7 parental (ATCC), but it is not clear whether or not this is due to differences in transfection efficiency between cells in regular media or charcoal stripped media or some other technical artifact (Fig. 13C).

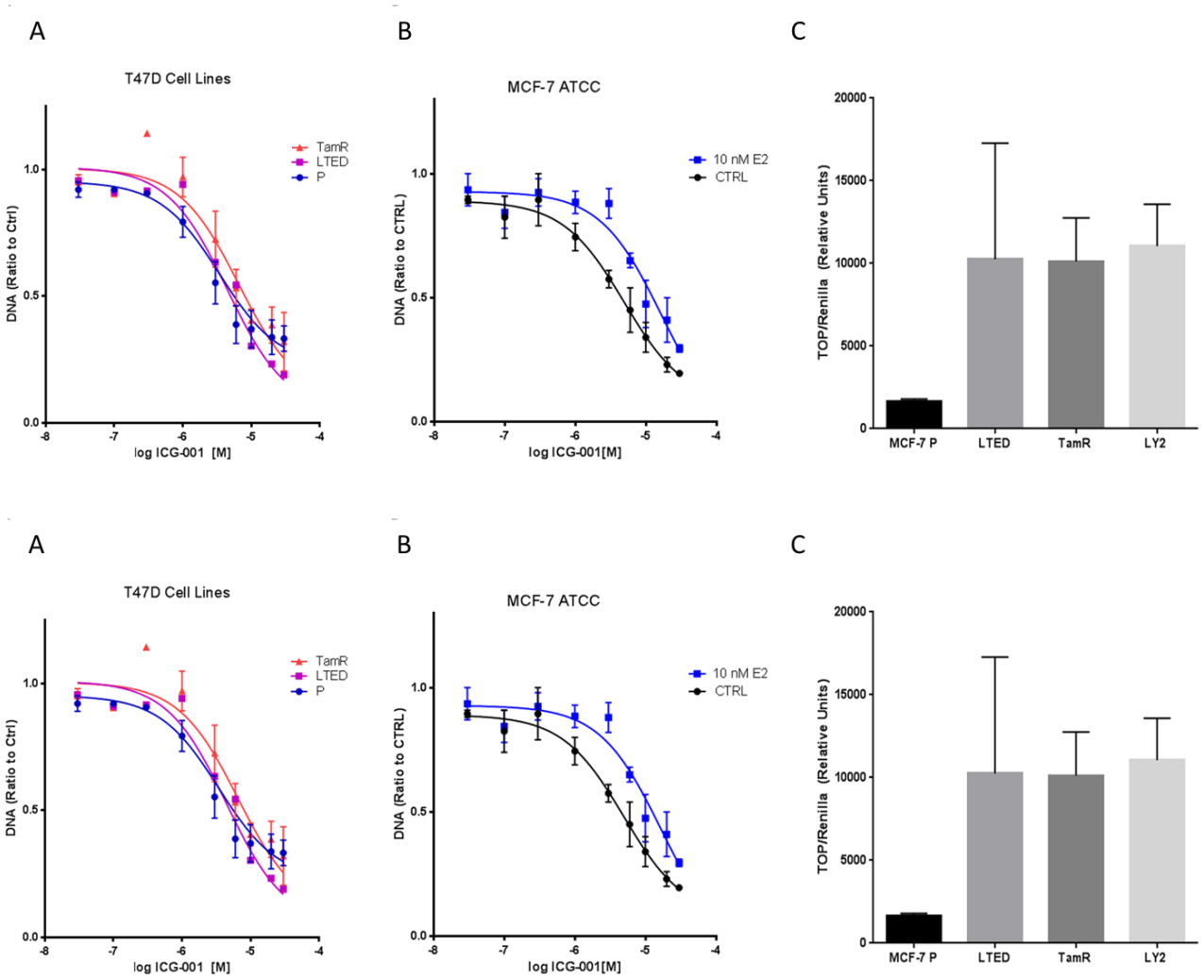

Figure 13. ICG-001 growth inhibition in endocrine sensitive cell lines. Cells were seeded onto 96 well plates at 3000 cells/well. 24 hours after plating, wells were treated with appropriate cell culture media for each cell line containing either vehicle control of 0.03-30 µM ICG-001. **A)** Growth of T47D Parental, T47D LTED and T47D TamR cell lines are all inhibited by ICG-001 with IC50s of 3.4, 4.2 and 6.1 µM respectively. **B)** MCF-7 ATCC cells are also sensitive to ICG-001; an addition of 10nM E2 shifts the IC50 from 4.9 µM to 17 µM. **C)** Preliminary data from TOP-Renilla luciferase assays in MCF-7 ATCC and MCF-7 LTED, TamR and LY2 cells.

2.3.7 β -catenin phosphorylation in parental and estrogen-independent MCF-7 cell lines

If Wnt signaling is upregulated in acquired endocrine resistance, one would expect to see significant differences in β-catenin levels between estrogen-dependent and estrogen-independent cells. To this end, we measured the basal levels of active and total β-catenin in the cell lysates of the estrogen-dependent and estrogen-independent cell lines using Western blotting techniques. Results showed no significant differences in the levels of active β-catenin between the various cell lines when compared to either total β-catenin or the loading control α-tubulin (Fig. 14 A-B).

Since β-catenin can also be activated by PKA and AKT, we tested for levels of β-catenin phosphorylated at these sites, S552, S675 (Fig. 14 C-D). Western blots showed no significant differences in the levels of any of other non-Wnt activated β-catenin.

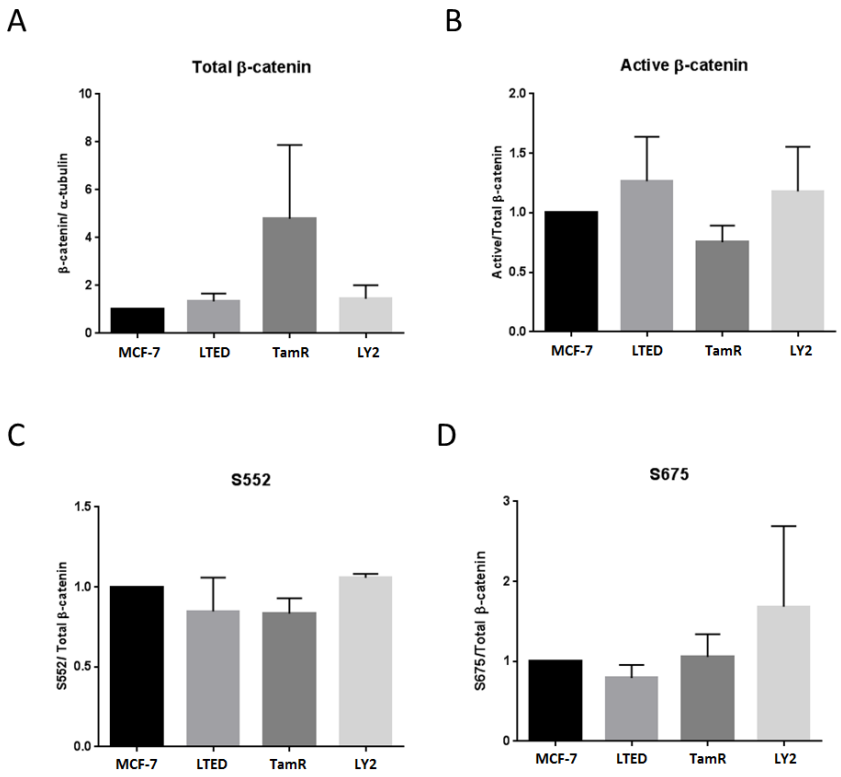

Figure 14. Western analysis of β-catenin expression in MCF-7 cell lines. Relative expression of total **(A)**, active **(B)**, S552 **(C)**, and β-catenin S675 **(D)** were determined using Western blotting techniques in MCF-7 ATCC and MCF-7 LTED, TamR and LY2 cell lines. ANOVA statistical analysis did not identify any significant changes in any of the experiments (n=3).

2.4 DISCUSSION

Several studies have implicated NHERF1 expression in breast cancer progression. Reports from out laboratory demonstrate that NHERF1 negatively regulates canonical Wnt signaling in the breast, implicating a novel mechanism for NHERF1 mediated breast cancer progression. However, unexpectedly, overexpression of WNT1A and WNT3A ligands in MCF10As did not

induce transformation. One explanation is that MCF10As could have downregulated expression of crucial Wnt/β-catenin signaling components, such as Dvl, Fzd or LRP5/6 receptors. Another explanation is that NHERF1 serves a redundant function in Wnt signaling that can be executed by other PDZ scaffold expressed in MCF10As. We next sought to determine if NHERF1 expression could be relevant in endocrine resistance. To model estrogen driven breast cancer, we used the estrogen-dependent MCF-7 and T47D cell lines and estrogen-independent cell lines derived thereof. Overall, the experiments conducted for this aim were inconclusive; the role of NHERF1 in the regulation of Wnt/β-catenin signaling in the breast remains enigmatic.

While the MCF-7 LTED, TamR and LY2s showed increased sensitivity to ICG-001 mediated growth arrest compared to the high passage MCF-7s, there were no significant differences in NHERF1 expression in two of the three estrogen-independent cell lines. This suggests that the apparent dependence on β-catenin mediated proliferation the LTED and TamR cell lines is not inhibited by the expression of NHERF1. Interestingly, Wnt conditioned media rescues MCF-7 cells from raloxifene but not toremifene induced growth inhibition. We identified toremifene as a SERM that does not significantly reduce NHERF1 expression in MCF-7s; nevertheless, it is possible the NHERF1 expression may not be the reason behind this phenomenon. As mentioned in chapter 1, SERMs can have different effects on signaling depending on the target tissue; SERMs cause conformational changes that influence ERα ability to bind to its co-activators or co-repressors, and the relative expression of these effectors determine the overall consequence of SERM treatment [323]. Other studies have demonstrated differences in tamoxifen, raloxifene and toremifene mediated changes in gene expression and enzyme induction [324]; it is possible that toremifene inhibits the expression of genes necessary

for Wnt-ligand mediated proliferation that are independent of NHERF1 expression. Further evidence is needed to determine which genes may be involved.

The observation that acute estrogen deprivation of parental MCF-7s significantly decreases NHERF1 protein levels, yet chronic estrogen deprivation in derived cell lines does not, was unexpected and in disagreement with the proposed model. This result suggests that upregulation of NHERF1 expression through non-estrogen mediated signaling pathways may present an advantage selected for in cells in culture. Therefore, it is possible that decreasing NHERF1 levels too low causes disadvantages to cell health and function that out-weigh any possible advantage caused by increases in Wnt/β-catenin activity. The increase of NHERF1 expression in certain samples of tamoxifen resistant recurrent breast cancer tumors suggests that, at least in some cases, NHERF1 expression can be up-regulated despite pharmacological inhibition of estrogen signaling. NFκB and mTOR signaling have both shown to increase NHERF1 expression, and upregulation of either of these signaling pathways could explain the unexpected high levels of NHERF1 [325, 326].

Additionally, results indicating that ICG-001 inhibits the proliferation of parental T47Ds and MCF-7 ATCC cells in the presence of estrogen were unexpected. We chose MCF-7s as our model for ER-mediated breast cancer for their documented sensitivity to the proliferative effects of E2 [327-329]. Similarly, others have reported that T47Ds are estrogen sensitive [330]. If the growth of estrogen-dependent cells can be inhibited by ICG-001, then β-catenin-mediated proliferation is not a phenotype exclusive to endocrine resistance. Indeed, evidence suggests that β-catenin and ERα interact genetically in *Drosophila* [331]. Furthermore, a recent study demonstrated that Wnt ligands can increase ERα expression, and that ERα and Wnt/β-catenin signaling act synergistically to promote the osteogenic differentiation of mesenchymal progenitor

cells [332]. Therefore, it is possible that in certain cell lines ERα could mediate some of its proliferative effects through crosstalk with β-catenin.

Human IHC studies of primary and recurrent breast cancer samples were inconclusive: one third of the samples showed the predicted decrease of NHERF1 expression and increase of activated β-catenin, another third showed the opposite trend, and the last third showed very little change. The main limitation of this study was the small sample size (n=6) and inability to attain more samples. Statistical power analysis (using G*Power software) indicates that, assuming up to 30% variation of samples, a total sample size of 20 would be necessary to achieve statistical significance (Matched pair test, effect size 0.8, α=0.05, and power of 0.95) .

The hypothesis predicted increased levels of activated β-catenin in estrogen-dependent cells; however, Western blotting results showed no differences in any of the forms of activated β-catenin between estrogen-dependent and estrogen-independent cell lines. Unfortunately, there was a lot of variation in the protein levels between sets of samples, which greatly contributed to the lack of statistically significant results and confounding some of the conclusions of this study. This variation could be explained by the use of the Promega Passive Lysis buffer to collect the samples for Western blotting. The recipe of the buffer is unknown, but it is possible that the detergent type or amount in the buffer did not allow for full solubilization of the nuclear compartment, explaining the large variations in β-catenin levels between data sets. Furthermore, it is important to note that at the time these studies were conducted it was unknown that certain strains of MCF-7 cells are sensitive to ICG-001 despite the presence of estrogen. Interestingly, preliminary data from TOP-Renilla luciferase assays indicate that the parental MCF-7 (low passage ATCC) cell used for the β-catenin Western blotting experiments have much less TCF/LEF signaling than their estrogen-independent counter-parts as determined by TOP-Renilla

experiments (Fig 13C), nevertheless ICG-001 still inhibits the proliferation of these cells even in media containing estrogen. Therefore, it is possible that the differences seen in basal TCF/LEF signaling as measured by TOP-Renilla reflect differences in transfection efficiency in estrogen-dependent and estrogen-independent cell lines, and not differences in levels of activated β-catenin. Finally, it is feasible that the observed effects of ICG-001 on proliferation could be explained by off-target effects of the drug that are independent of β-catenin signaling. However, no such off-target effects have been reported for ICG-001 thus far, and more data is needed to determine if this is indeed a possibility.

3.0 THE NA^+/H^+ EXCHANGER REGULATORY FACTOR 1 (NHERF1) REGULATES WNT SIGNALING AND CILIOGENESIS IN EPENDYMAL CELLS

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Multi-ciliated cells generate vectorial fluid flow in the brain and other organs. Deficiencies in the formation and organization of cilia result in developmental anomalies including hydrocephalus. Ciliary organization is linked to the Planar Cell Polarity (PCP) signaling pathway. Here we report that mice lacking the Na^+/H^+ Exchanger Regulatory Factor 1 (NHERF1, also known as EBP50) develop communicating hydrocephalus associated with disorganized and dysfunctional motile cilia in the ependyma. This defect is due to disruption of the Wnt signaling pathways in the absence of NHERF1. We show that NHERF1 binds the PCP core genes Frizzled (Fzd) and Vangl. We further show that NHERF1 promotes translocation of Vangl2 to the plasma membrane of cells in culture and to the apical surface of ependymal cells. Furthermore, NHERF1 assembles a ternary complex containing Fzd4 and Vangl2. These results demonstrate that NHERF1 plays a critical role in the development of functional motile cilia.

3.1 INTRODUCTION

Ciliopathies constitute a growing class of genetic diseases with clinical manifestations that include neurodevelopmental defects, central nervous system (CNS) anomalies, laterality defects, and congenital heart disease [333]. Ciliary dysfunction resulting from one or more mutations in genes that regulate the assembly or function of primary, sensory, or motile cilia is commonly shared as the origin of these syndromes. Hydrocephalus is frequently associated with genetic ciliary dysfunction as a consequence of abnormalities in the ependyma, a layer of ciliated polarized epithelial cells that differentiate from radial glia to form the lining of the cerebral ventricles [334]. Ependymal cilia beat in a coordinated fashion that promotes the circulation of cerebrospinal fluid (CSF). Mutations in genes involved in the assembly and structure of ependymal cilia affect CSF dynamics resulting in hydrocephalus [335-338]. The genetic factors that govern ciliary development and function in the ependyma remain poorly understood. Recent work links ependymal ciliogenesis to non-canonical Wnt signaling, specifically to the Planar Cell Polarity (PCP) pathway [198, 199].

NHERF1 is a member of the PSD-95/Discs-large/Zo-1 (PDZ) family of proteins. NHERF1 contains two N-terminal PDZ domains and a C-terminal Ezrin/Radixin/Moesin/Merlin-binding domain (EBD) that attaches to the cytoskeleton [48]. Hydrocephalus has been noted in NHERF1 knockout animals [39]. The origin of this phenotype is uncertain. We show here that the ependymal epithelium of NHERF1$^{-/-}$ mice contains dysfunctional cilia as a consequence of altered Wnt signaling.

3.2 MATERIALS AND METHODS

Reagents and Materials: Primary antibodies for HA were purchased from Covance. Anti-Vangl2 antibodies were from Abcam. Anti-NHERF1 antibodies were purchased from Santa Cruz. Anti-GFP antibodies were from Clontech. All secondary antibodies were purchased from Jackson Immunoreagents. X-tremeGENE HP transfection reagent was purchased from Roche. Opti-MEM and Ham's F-12 media were purchased from Life technologies. All other reagents used were purchased from Sigma. HA-tagged human Fzd4 was kindly provided by Dr. T. Kirchhausen. HA-tagged rat Fzd1 was a generous gift from Dr. R. Habas. Vangl2 was purchased from Addgene and subcloned downstream of EGFP. Vangl1 and Vangl1ΔPDZ were a gift from Dr. P. Gros. HA-Vangl2 was a gift from Dr. D. Ginty.

Immunoprecipitation and immunoblot: CHO-N10 cells stably expressing Fzd4 were transiently transfected with GFP-Vangl2 or empty vector. NHERF1 expressions was induced with 50 ng/ml tetracycline and after 48 h the cells were lysed with RIPA buffer supplemented with protease inhibitors and incubated for ice for 15 min. Lysates were incubated overnight at 4°C with HA.11 monoclonal affinity matrix (Covance). Total lysates and immunoprecipitated protein were analyzed by SDS-polyacrylamide gels and transferred to Immobilon-P membranes. The blots were probed with the following specific antibodies: NHERF1 (Santa Cruz), GFP (Life technologies), and HA.11 (Covance). All primary antibodies were used at a concentration of 1 μg/ml.

Live cell imaging/Vangl2 localization–CHO N10 cells were transfected with HA-Fzd1 and either EGFP-Vangl2 or EGFP-V521A-Vangl2. Twenty-four h post transfection cells were treated with vehicle or 50 ng/ml tetracycline to induce NHERF1 expression and incubated for

another 24-48 hr. Live cells were decorated with Covance HA.11 primary antibody for 30 min at room temperature, rinsed with PBS and then incubated with 2 μg/ml goat anti-mouse Alexa fluor 546 (Life technologies) conjugated secondary antibodies for 30 min. After rinsing, the cells were imaged using a confocal microscope. The cross correlation of HA-Frizzled 1 and GFP-Vangl2 or GFP- V512A-Vangl2 was measured using Image J software.

Image Cross-Correlation Spectroscopy: CHO N10 cells transfected with GFP-Vangl2 and HA-Fzd4 were treated with 50 ng/ml tetracycline to induce NHERF1. Live cells were incubated Alexa fluor 594-conjugated Covance HA.11 antibodies (2 μg/ml) for 30 min at room temperature to label Fzd4 receptors at the plasma membrane. Cells were rinsed and then imaged using an Olympus Fluoview1000 confocal microscope. To measure the autocorrelation and cross-correlation functions, the microscope was focused at the plasma membrane, and 100-200 frames of a small area (30x30 pixels) were obtained by continuous scanning at a rate of 30 ms/frame. The autocorrelation and cross-correlation functions of EGFP-Vangl2 and HA-Fzd4 were calculated using an ImageJ plugin specifically written for this purpose.

Fluorescence recovery after photobleaching (FRAP): CHO N10 cells were transfected with HA-Fzd4 and EGFP-Vangl2 and labeled as previously described and incubated with HA.11 anti-HA antibodies(see [18,19]). A subset of cells from each group was incubated with 5 μg/mL goat anti-mouse secondary antibody for 30 min at room temperature to immobilize Fzd4 receptors. All FRAP measurements were done focusing on the plasma membrane adjacent to the coverslip. Cells that expressed EGFP-Vangl2 at the plasma membrane were identified and circular regions of interest were selected and bleached with the 488-nm laser line using a Fluoview 1000 equipped with a SIM scanner. EGFP fluorescence recovery of the bleached area

was recorded over time. The data were fitted to a single exponential decay using GraphPad Prism and the immobile fraction of each group was determined.

Immunohistochemistry: The brains of 4-10 week old animals ($NHERF^{-/-}$ and wild-type littermates) were fixed in 4% formalin for 48 h at 4°C, dehydrated, embedded in paraffin and sectioned into 5μm slices using a microtome. For immunohistochemistry, glass slide mounted slices were rehydrated by successive washes with xylene, 100% ethanol, 95% ethanol, 75% ethanol, and PBS. Antigen retrieval was performed by heating the slides in citrate buffer. Samples were blocked with 5% BSA and exposed to primary antibody (2 μg/ml) at 4°C overnight. Slides were probed with various primary antibodies (see table 4). The slides were washed and incubated with the appropriate secondary antibody (dilution 1:1000) for 2 h at room temperature, further stained with DAPI for 5 min, washed briefly, and covered with a coverslip. All tissues were examined with an Olympus Fluoview 1000 confocal microscope.

Transmission Electron Microscopy: Brains were harvested and immersion fixed in 2.5% glutaraldehyde overnight at 4^oC. Brains were sectioned such that ependymal epithelium was revealed on a surface of the tissue slice. Following fixation, tissue washed 3x in PBS then post-fixed in aqueous 1% OsO_4, 1% $K_3Fe(CN)_6$ for 1 h. Following 3 PBS washes, the tissue was dehydrated through a graded series of 30-100% ethanol, 100% propylene oxide then infiltrated in 1:1 mixture of propylene oxide:Polybed 812 epoxy resin for 1 h. After several changes of 100% resin over 24 h, brain slices were embedded in molds, cured at 37^oC overnight, followed by additional hardening at 65^oC for two more days. Ultrathin (60 nm) sections of tissue were collected on copper grids, stained with 2% uranyl acetate in 50% methanol for 10 min, followed by 1% lead citrate for 7 min. Sections were imaged using a JEOL JEM 1210 transmission electron microscope at 80 kV fitted with a side-mount digital camera.

Scanning Electron Microscopy: Mouse tissues were processed as for TEM above, but 1-mm thick longitudinal slices that reveal the surface epithelium were used. Tissue was processed up to the final 100% ethanol, then chemically dried using hexamethyldisilazane. Dried slices were mounted onto aluminum stubs, grounded with silver paint then sputter coated with 3.5 nm gold/palladium (Auto 108, Cressington, and Watford, UK). Samples were viewed in a JEOL JSM-6330F scanning electron microscope (Peabody, MA) at 3 kV. *Ciliary Function Assay–* Fresh mouse brains were sectioned into 1 mm slices using a hand held slicer (Zivic Instruments) and mounted onto a glass-bottomed chamber filled with clear MEM. To measure ciliary function, 2 μl of a suspension of 1 μm fluorescein-tagged beads were added to the observation space (the third ventricle) and the motion of the fluorescent beads was monitored using a microscope equipped with a 20X water immersion objective. Time courses of up to 60 sec were recorded and bead velocity was determined using ImageJ. For tracheal tissue, the tracheae were excised, cut longitudinally and mounted with the tracheal epithelium exposed. The fluorescent beads were added over the exposed surface and bead motion was examined as described.

Nherf1 Gene Knockdown in Zebrafish: All zebrafish experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Embryos were obtained from wildtype (AB*) through natural mating. *NHERF1 (SLC9A3R1)* antisense morpholinos *(5'-CCTGAGGTCGCTGGACATTTT-3')* (NHERF1-MO) were designed and synthesized by GeneTools, LLC (Philomath, OR). MO concentrations ranging from 1-10ng were injected into the 1-cell stage embryos as previously described [339]. Embryos were incubated to the desired stage, then directly imaged under a Leica stereomicroscope and photographed using a digital camera.

Statistical analysis: All experiments were repeated at least three times. GraphPad PRISM was used for all statistical analyses

Table 3. Plasmids and constructs used in Chapter 3

Plasmid/Construct	Vector	Source
EGFP-Vangl1	pCS2	P. Gros laboratory
EGFP-Vangl1 ΔPDZ	pCS2	P. Gros laboratory
EGFP-Vangl2	pcDNA3.1+	G. Romero laboratory (from EGFP and Vangl2 Addgene vectors)
EGFP-Vangl2 V521A	pcDNA3.1+	G. Romero laboratory (site directed mutagenesis)
HA- Fzd4	pcDNA3	T. Kirchhausen laboratory
HA-Fzd1	pcDNA3.1+	Cloned from pCS2+ vector provided by R. Habas laboratory
HA-Vangl2	pCS2+	D. Ginty laboratory
NHERF1 morpholinos		GeneTools, LLC (Philomath, OR)

Table 4. Antibodies used in Chapter 3

Antibody	**Type**	**Species**	**Source**
anti-hamster Alexa 488	secondary	Goat	Abcam ab173003
anti-rabbit Alexa 594	secondary	Goat	Life technologies A-11012
anti-mouse Alexa 488	secondary	Goat	Life technologies A-11001
anti-mouse Alexa 546	secondary	Goat	Life technologies A-11030
anti-mouse HRP	secondary	Goat	BioRad 1721011
anti-rabbit HRP	secondary	Goat	BioRad 1706515
alpha-tubulin	primary	Rabbit	Abcam ab4074
β-catenin	primary	Rabbit	Millipore 06-734
GFP	primary	Rabbit	Life technologies A-6455
HA.11	primary	Mouse	Covance 16B12 MMs-101P
HA.11 conjugated matrix	primary	Mouse	Covance 16B12 AFC-101P
NHERF1	primary	Rabbit	Santa Cruz H-100 sc-134485
NHERF1	primary	Rabbit	Pierce antibodies PA5-17044
Mucin-1	primary	Ar. Hamster	ThermoFisher Sci MA5-11202
Vangl2	primary	Mouse	Pierce antibodies PA5-18654

3.3 RESULTS

3.3.1 NHERF1 $^{-/-}$ mice develop communicating hydrocephalus

The mice used in these studies were derived from the NHERF1$^{-/-}$ clones developed by Shenolikar et al [39] after back breeding with C57BL/6 for 10 generations to produce an isogenic line. About 35% of the NHERF1$^{-/-}$ mice showed clinically relevant hydrocephalus (Fig. 15A-B) between 28 and 32 days after birth. Ventricular dilation was detected in all animals examined (n=12), independently of whether or not external signs of hydrocephaly were visible. Dilation of the cerebral ventricles was not found in any of the wild type and in only one of the heterozygote animals. Detailed examination of brain slices from severely hydrocephalic NHERF1$^{-/-}$ animals did not reveal obstructions in the ventricles, arachnoid granulations, or the cerebral aqueduct, suggesting that the syndrome is a form of communicating hydrocephalus. To confirm that NHERF1 knockdown is sufficient to cause hydrocephalus, we injected 1-cell stage zebrafish (*Danio rerio*) embryos with antisense morpholino oligonucleotides targeted to the nherf1/Slc9a3r1 initiation codon (NHERF1-MO). All injected embryos showed impaired balance and motility accompanied by severe hydrocephalus and cardiac edema at 48 h post fertilization (hpf) (Fig. 15C). This phenotype was accompanied by a shortened longitudinal axis and defects in tail formation (Fig. 15D) reminiscent of the Wnt5a/pipetail phenotype.

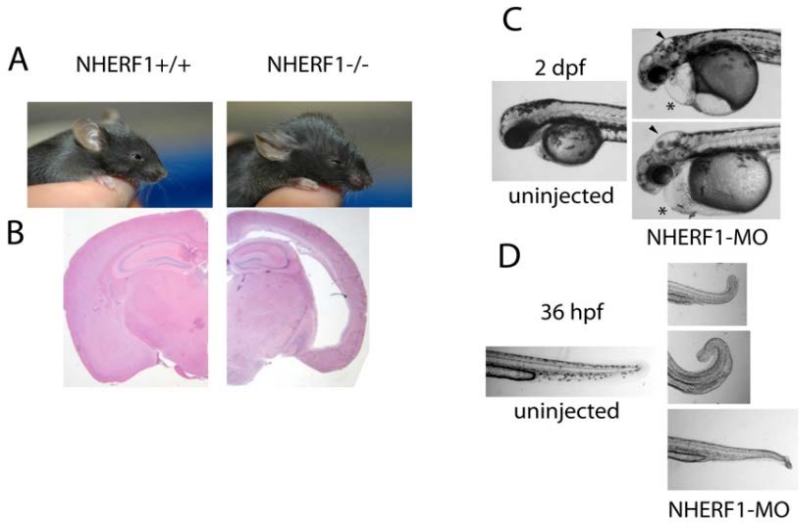

Figure 15. NHERF1$^{-/-}$ mice display hydrocephalus. A) Comparison of two 28-day old NHERF1+/+ (left) and NHERF1$^{-/-}$ (right) littermates. B) Nissl-stained coronal sections from NHERF1+/+ (left) and NHERF1-/- mice at P28. C) Depletion of nherf1 in zebrafish embryos produced pronounced hydrocephalus (arrows) and cardiac edema (*) 2 days post-fertilization. D) NHERF1 knockdown also resulted in tail developmental defects. Data for sections A &B were collected by David Wheeler.

3.3.2 Ciliary defects in NHERF1 $^{-/-}$ mice

We examined the expression of NHERF1 in the ependyma and choroid plexus of wild-type mice. NHERF1 is abundantly expressed in the ependymal epithelium (Fig. 16A). Because of the high levels of NHERF1 in ependyma, and given that impaired ciliary function is a frequent cause of hydrocephalus [335-338], we stained ependymal cilia by staining with acetylated α-tubulin antibodies (Fig. 16B). Compared to wild type animals, the number of cilia was reduced in NHERF1$^{-/-}$ mice (Fig. 16B). Many ependymal cells had fewer cilia, and some had none. A similar phenomenon was observed in the otic vesicles of zebrafish embryos injected with NHERF1-MO (Fig. 16C). The number of cilia present in the cristae of the otic vesicles of

NHERF1-MO injected embryos were reduced in 38 of the 42 specimens examined (Fig. 16C). Importantly, NHERF1-MO injections did not disrupt the actin cap of the otic vesicle epithelium, suggesting that NHERF1 knockdown had no effects on the apical-basolateral polarization of the cells. Scanning electron microscopy studies confirmed the tubulin staining data, demonstrating a significant reduction in the number of cilia in NHERF1$^{-/-}$ mice. The images further showed significant differences in the orientation of the cilia, with the ciliary tufts of adjacent cells often pointing in opposite directions (Fig. 16D). Importantly, the absence of NHERF1 did not alter the differentiation of ependymal cells as measured by the expression of ependymal markers such as S100β [334].

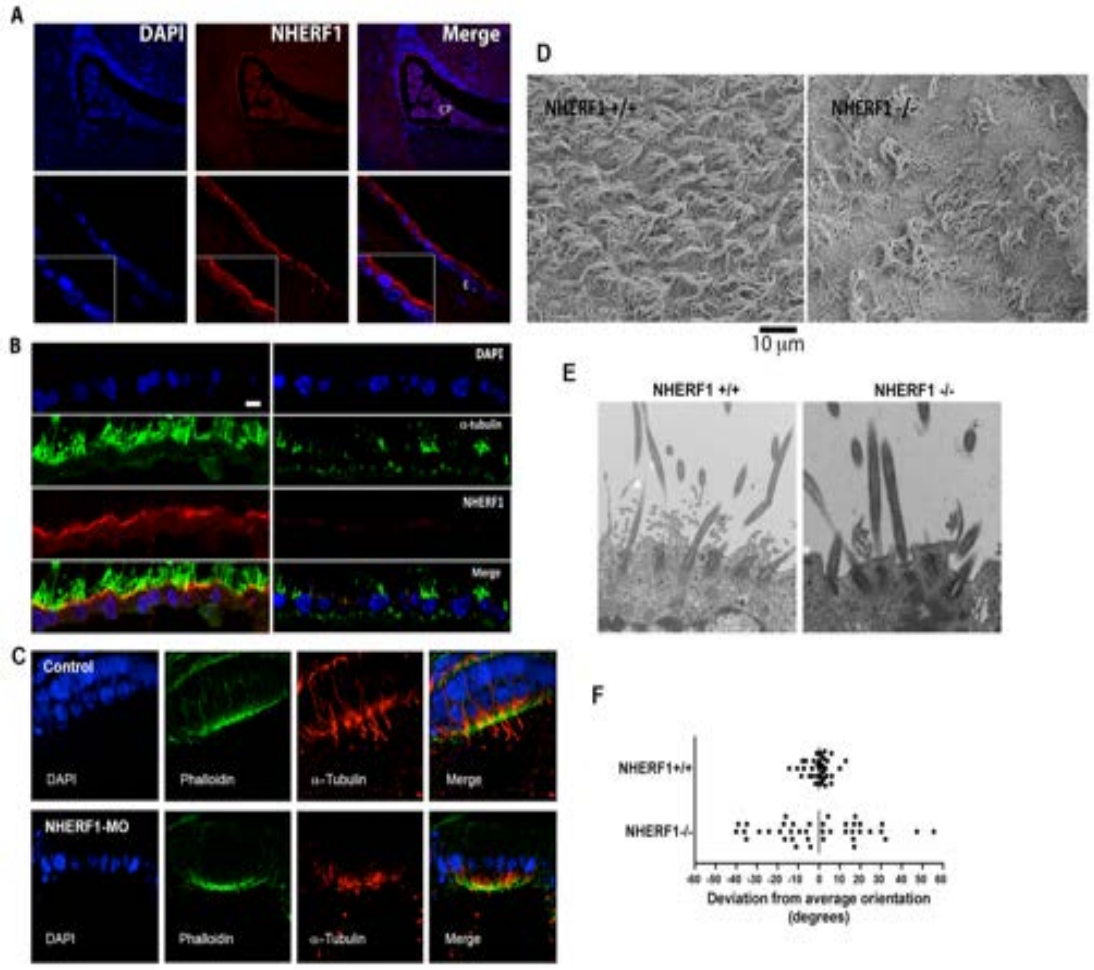

Figure 16. NHERF1 expression in the ependyma. A) Immunofluorescence staining of a sagittal section of the third ventricle of a NHERF1$^{+/+}$ mouse. CP: choroid plexus; E: ependymal layer. Sagittal sections of paraffin embedded P28 wild type mouse brain were stained with anti-NHERF1 antibody followed by a TRITC-labeled secondary antibody. The sections were then imaged using a confocal microscope. **B)** Ciliary defects in the NHERF1$^{-/-}$ mouse**.** Sagittal sections of the ependyma of P28 wild type and NHERF1$^{-/-}$ mice were stained with antibodies specific for acetylated α-tubulin and NHERF1. The bar represents 10 μm. **C)** Ciliary defects in the otic vesicle caused by injection of NHERF1-MO**.** Whole embryos (48 hpf) were fixed in 4% PFA, stained with phalloidin and anti-α-tubulin and the otic vesicles were examined by confocal microscopy. **D)** Scanning electron micrograph of the ependyma of wild type and NHERF1$^{-/-}$ mice. **E)** Defective orientation of ependymal cilia in the NHERF1$^{-/-}$ mouse**.** Transmission electron micrographs of the surface of ependymal cells from P28 wild type and NHERF1$^{-/-}$ mice. **F)** Comparison of the ependymal ciliary orientation in NHERF1$^{+/+}$ and NHERF1$^{-/-}$ mice. To measure the orientation of the cilia, the angles formed by the basal body of each cilium and the tangent to the cell surface (θ) were measured and averaged for all cilia in each field. The deviation from the average orientation was determined for each individual cilium as θ-$\theta_{Average}$. Data for sections A & B were generated by David Wheeler, sections D-F were generated by Donna Stoltz.

To evaluate the orientation of the ependymal cilia, we performed transmission electron microscopy experiments and measured the relative angles of the basal bodies of the cilia with the surface of the cell (Fig. 16E, 16F). This analysis demonstrated that, whereas the basal bodies of the wild type animals were roughly oriented in the same direction (± 5 degrees), those of the NHERF1$^{-/-}$ animals were disorganized, pointing in random directions (Fig. 16E).

The ultrastructure of the residual cilia found in NHERF1$^{-/-}$ mice was unremarkable. The cilia displayed the typical 9+2 structure of normal motile cilia, and individual cilia were of approximately the same length and diameter when compared to the wild type ependyma (Fig. 17). This suggests that NHERF1 is not structurally involved in the architecture of the cilia. Thus, NHERF1 ablation causes reduced number and altered orientation of the cilia but does not alter the structural organization of the cilium.

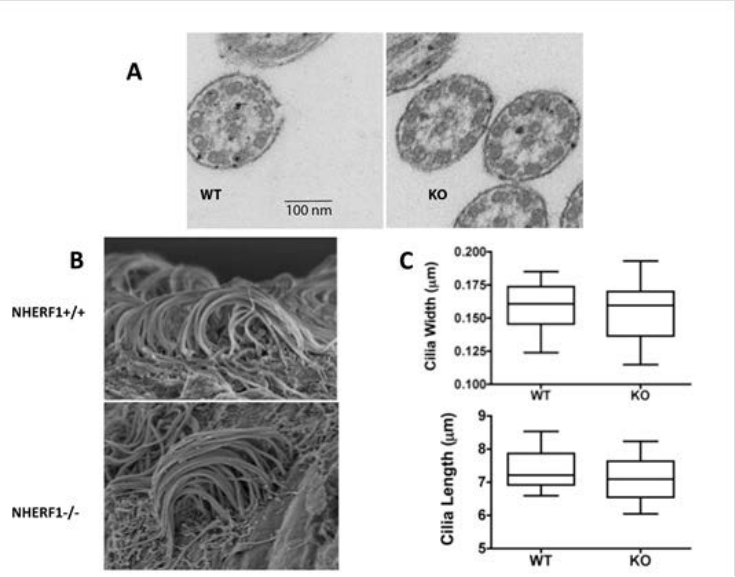

Figure 17. Ultrastructure of the ependymal cilia. A) Transmission electron micrographs of the motile cilia of NHERF1$^{+/+}$ and NHERF1$^{-/-}$ does not reveal any differences in architecture. **B-C)**, Scanning electron microscopy does not reveal any differences in length and diameter between NHERF1$^{+/+}$ and NHERF1$^{-/-}$ ependymal cilia**.** Data for this figure was collected by Donna B. Stoltz.

The cross-species hydrocephalus phenotype suggests defects with vital functional consequences. Therefore, we examined the ability of the ependymal cilia to generate hydrodynamic fluid movement. This was done using 1 μm fluorescein-labeled latex beads placed onto the third ventricle of freshly prepared brain slices from 28-day old mice (Fig. 18A). The fluorescent beads moved rapidly and in an elliptical trajectory in the wild type slice preparations (average velocity =18.2 ± 2.2 μm/s). In contrast, there was little discernable movement in the NHERF1$^{-/-}$ slices (average velocity = 1.1±1 μm/s) (Fig. 18B), which was statistically indistinguishable from the Brownian motion of the beads in the azide-poisoned brain slices that served as a negative control.

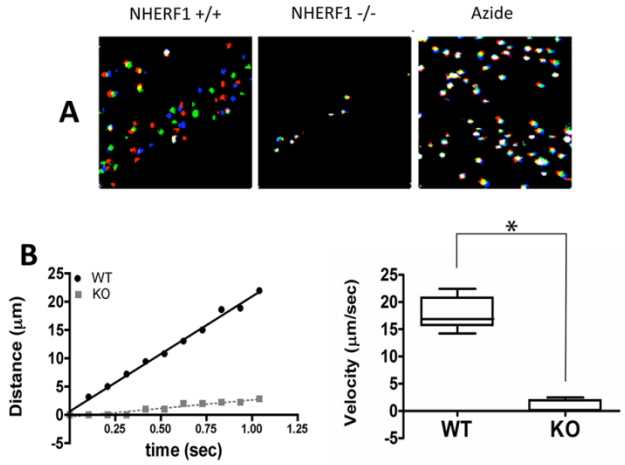

Figure 18. The cilia of NHERF1$^{-/-}$ ependyma are dysfunctional. A) Ciliary function was determined by measuring the motion of 1 μm fluorescein-labeled beads placed within the third ventricle of sagittal brain slices obtained from P28 wild type and knockout mice. To illustrate the motion, 3 successive images (Δt=0.1 s) were colored red, green and blue, respectively, and combined in ImageJ. The image on the left, characteristic of the wild type brain slices, shows no superposition of the three colors, indicating rapid motion of the beads. The beads of the NHERF1$^{-/-}$ slices appear white in the montage because all three colors (red, green and blue) coincide at all times, indicating lack of motion. The right side panel (Azide) shows the results obtained with a wild type slice poisoned with azide. Refer to Supplementary Movie SM1 for complete visualization of the experiment. **B)** Tracking of individual beads in wild type (WT) and knockout (KO) mice calculated as described. The right panel shows average velocities estimated from the tracking of individual beads. (*) denotes statistically significant differences ($p<0.001$;

results obtained from three independent experiments). Data for this figure was generated by David Wheeler and Guillermo Romero.

3.3.3 NHERF1 regulates Wnt signaling

Although these results imply that NHERF1 is required for the formation of functional cilia, NHERF1 is excluded from the cilium and is not essential for the formation of primary cilia [340], suggesting that NHERF1 does not play a structural role in cilia formation. Therefore, we turned our attention to the signaling pathways involved in the development of functional motile cilia. A well-documented regulator of motile cilia is the Planar Cell Polarity (PCP) signaling pathway [198, 199]. Ablation of two of the PCP core genes, Celsr/Flamingo and Van Gogh/Vangl, causes dysfunction of motile cilia and a hydrocephaly phenotype similar to the one we describe [198, 199]. Furthermore, we recently showed that NHERF1 interacts directly with Frizzled receptors and negatively modulates canonical Wnt signaling in breast cancer cell cultures and in murine breast ducts [40]. Therefore, we hypothesized that the effects of NHERF1 ablation on ciliary development are a consequence of NHERF1's function in Wnt and PCP signaling.

We measured nuclear β-catenin levels in brain slices of wild type and NHERF1-/- animals to test the role of NHERF1 in the coupling of Wnt signaling in the ependyma. β-catenin levels were higher in the nuclei of knockout animals (Fig. 19A). Because Vangl2 plays a crucial role in the development of tissue polarity and contains a typical PDZ binding motif in its C-terminus, we examined the effects of NHERF1 ablation on the expression and subcellular distribution of Vangl2. In normal ependyma, Vangl2 localizes to the apical surface of the cells in close proximity to the cilia [341]. We found substantial co-localization of NHERF1 and Vangl at

the apical surface of the ependyma of wild type animals. In contrast, Vangl2 expression was reduced and miss-localized in the ependyma of the NHERF1$^{-/-}$ mice (Fig. 19B). Importantly, mucin-1 staining was comparable in wild type and knockout animals, suggesting that the miss-localization of Vangl2 was not caused by defects in the apical-basolateral polarization of the ependymal epithelium (Fig. 19C).

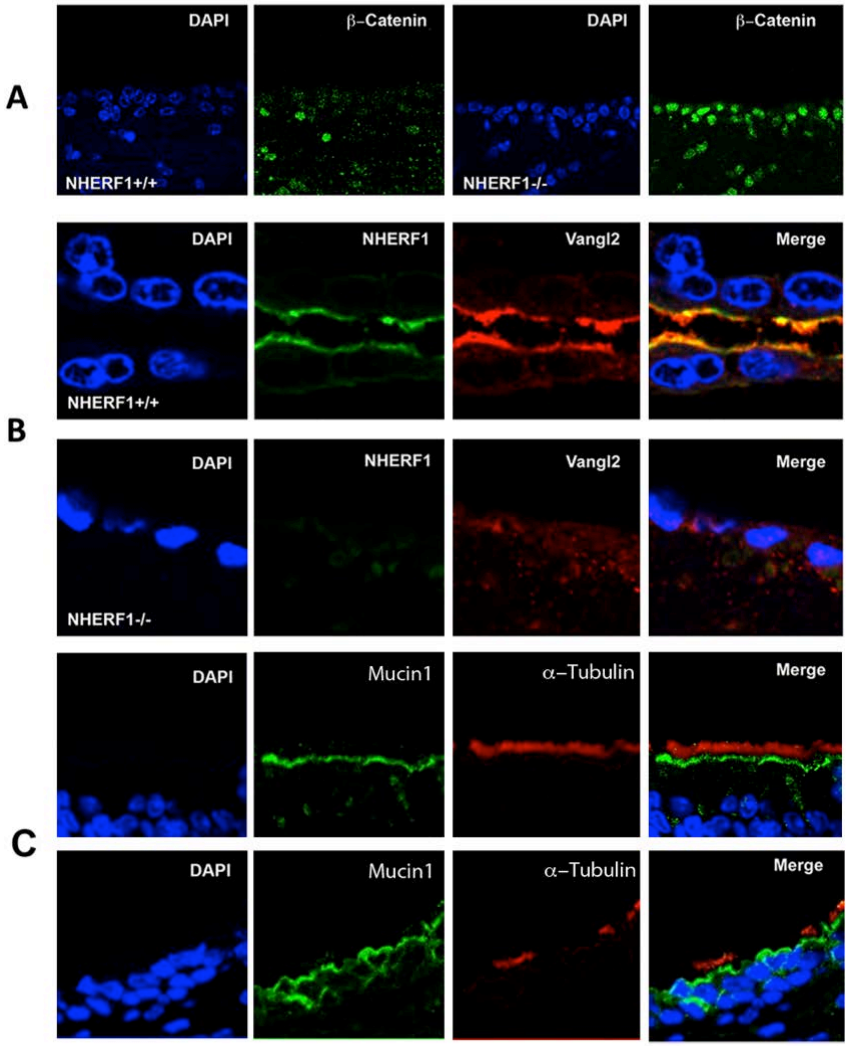

Figure 19. Abnormal canonical and non-canonical Wnt signaling in the ependyma of NHERF1$^{-/-}$ mice. A) Increased nuclear β-catenin levels in ependyma of NHERF1$^{-/-}$ mice. Brain slices from NHERF1$^{+/+}$ and NHERF1$^{-/-}$ mice were fixed and stained with a monoclonal antibody that recognizes the activated form of β-catenin. **B)** Vangl2 mislocalization in NHERF1$^{-/-}$ ependyma. Slices showing the cerebral aqueduct of from NHERF1$^{+/+}$ (upper panels) and NHERF1$^{-/-}$ (lower panels) were stained with specific anti-NHERF1 and anti-Vangl2 antibodies. The second row of nuclei is absent in the NHERF1$^{-/-}$ sample because of the dilation of the aqueduct as a consequence of hydrocephalus. **C)** Defective ciliogenesis is not accompanied by gross defects in the apical-basolateral polarization of the ependymal epithelium. Brain sections showing the third ventricles of wild-type and knockout animals were stained with mucin-1 and α-tubulin antibodies. Notice normal expression of mucin-1 in the ependyma of NHERF1$^{-/-}$ animals independently of the presence of cilia. Data for section A was collected by David Wheeler.

3.3.4 PDZ domain-PDZ ligand interactions regulate the traffic and function of Vangl2

To further examine the role of NHERF1 in Vangl2 traffic, we transfected CHO cells that express NHERF1 in a tetracycline-dependent manner (CHO-N10) with HA-tagged-Fzd1 and EGFP-tagged-Vangl2 constructs. In the absence of tetracycline (*i.e.*, no NHERF1), the Vangl2 construct was expressed primarily in cytosolic structures (Fig. 20A). Addition of tetracycline increased the plasma membrane levels of EGFP-Vangl2, where it co-localized with Fzd1 (Fig. 20A, 20C). We hypothesized that NHERF1 regulates Vangl localization and traffic by direct interactions with the Vangl C-terminal PDZ binding motif. To test this hypothesis, we transfected CHO-N10 cells with EGFP-V521A-Vangl2, a mutant in which the C-terminal valine has been replaced by alanine, thus disrupting its binding to PDZ scaffolds. The mutant Vangl2 was retained in cytosolic vesicles, independently of the expression of NHERF1 (Fig. 20B, 20*C*). Furthermore, equivalent results were obtained with Vangl1, which harbors an identical C-terminal PDZ binding motif (Fig. 21). Therefore, an intact PDZ binding motif in Vangl1/Vangl2 is required for correct localization.

Because NHERF1 contains two PDZ domains, we investigated the effects of mutations of each individual PDZ motif on the distribution of Vangl2. CHO cells were transfected with EGFP-Vangl2 and Flag-tagged NHERF1 constructs harboring mutations in the core of one or both PDZ domains (S1: mutated PDZ-1: S2: mutated PDZ2; S1S2: both PDZ domains mutated). Co-transfection with wild-type NHERF1 promoted limited plasma membrane localization of Vangl2; in contrast, plasma membrane localization was not detected when any of the PDZ domain mutants were co-transfected with EGFP-Vangl2 (Fig. 20D). Interestingly, the effects of wild type NHERF1 on the plasma membrane expression of EGFP-Vangl2 were much greater in cells that had been transfected with Frizzled receptors (compare Fig. 20A and Fig. 20D). Therefore, we hypothesized that the expression of Vangl2 at the plasma membrane required the formation of ternary complexes containing NHERF1, Vangl2 and Fzd. To confirm the formation of these complexes, we performed co-immunoprecipitation experiments in CHO-

N10 cells transfected with HA-tagged Fzd4 and EGFP-Vangl2. We found that, as predicted, NHERF1 co-immunoprecipitated with Fzd4 and Vangl2 (Fig. 22A, 22B). We also observed a weak interaction between Vangl2 and Fzd4 that was significantly enhanced by the expression of NHERF1 (Fig. 22C).

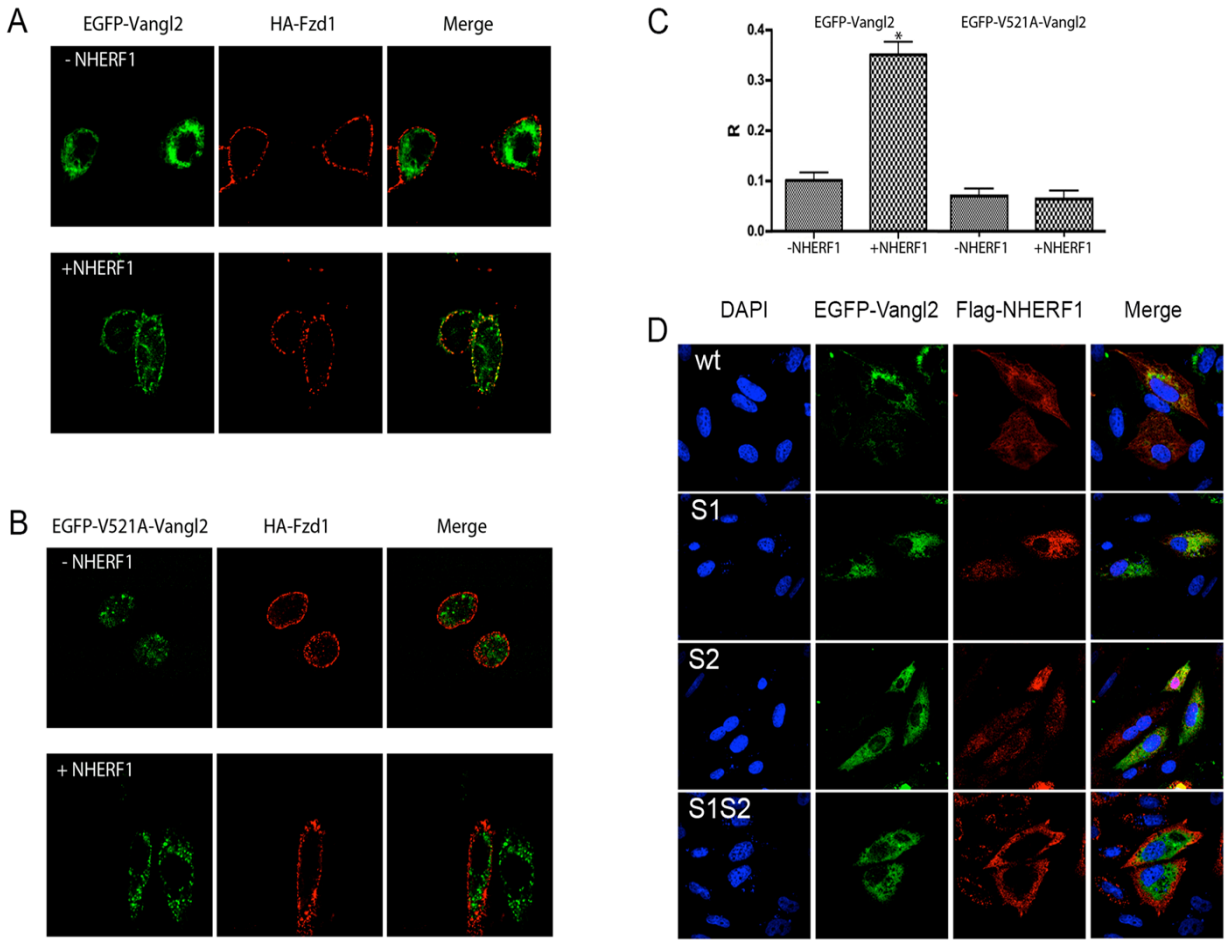

Figure 20. NHERF1 promotes plasma membrane localization of Vangl2. A) CHO-N10 cells expressing NHERF1 in a tetracycline-sensitive manner were transfected with HA-Fzd1 and EGFP-Vangl2. The cells were treated with 50 nM tetracycline or vehicle, decorated with anti-HA antibody followed by Alexa 594-labeled secondary antibody, and examined by confocal microscopy. **B)** Same as **A** except that the cells were transfected with an EGFP-tagged Vangl2 construct in which the C-terminal PDZ binding motif has been mutated to preclude binding to PDZ domains (V512A-Vangl2). **C)** Co-localization of HA-Fzd1 and EGFP-Vangl2. The co-localization was quantified using the Spearman correlation coefficient measured using ImageJ. The correlation coefficients shown represent the average obtained from >50 cells for each set, examined in six separate experiments. The symbol (*) denotes statistically significant differences ($p<0.001$). **D)** NHERF1-induced plasma membrane localization of Vangl2 requires both PDZ domains. CHO cells co-transfected with Flag-tagged NHERF1 constructs and EGFP-Vangl2 were fixed, decorated with anti-Flag antibodies and examined with a confocal microscope. Only the wild type NHERF1 construct promoted plasma membrane localization of Vangl2. Wild-type NHERF1 co-localized with Vangl2; reduced co-localization was observed with the S1 and S2 mutants, whereas the S1S2 mutant did not co-localize with NHERF1 at all.

Because NHERF1 contains two PDZ domains we hypothesized that NHERF1 promotes the formation of Fzd4-NHERF1-Vangl2 ternary complex through its interactions with the C-terminal PDZ binding motifs of Fzd4 (–ETVV) and Vangl2 (–ETSV). To test this hypothesis, we co-transfected HEK293S GnTI cells (which contain low levels of NHERF1 but express abundant endogenous Fzd4) with HA-tagged Vangl2 and FLAG-tagged NHERF1 constructs (wild-type, S1, S2 and S1S2). We subsequently immunoprecipitated HA- Vangl2 and detected the presence of endogenous Fzd4 in the immunoprecipitate by Western blotting. The results shown in Fig. 22D indicate that NHERF1 requires both PDZ domains to promote the formation of Fzd4-Vangl2 complexes. These data are consistent with the formation of NHERF1–Fzd4–Vangl2 ternary complexes by a mechanism that involves the interactions of the C-terminal PDZ ligands of Vangl2 and Fzd4 with the PDZ domains of NHERF1. We further studied the formation of these complexes by optical methods using an N-terminal-EGFP-tagged Vangl2 construct and Fzd4 tagged with an HA epitope near the N-terminus such that it is exposed to the extracellular milieu (Fig. 23A). These cells were incubated with Alexa594-tagged anti-HA antibody specifically to label the HA-Fzd4 exposed to the membrane. In one set of experiments, the HA-tagged Fzd4 was immobilized at the membrane by addition of a crosslinking secondary antibody and the mobility of the EGFP-Vangl2 construct was determined by FRAP. Figure 23B shows that the addition of the crosslinking antibody decreases the mobility of Vangl2 only in cells that express NHERF1. We further examined formation of ternary complexes using image cross-correlation spectroscopy (ICCS), a technique that measures complex formation by determining the correlation of the intensity fluctuations of differentially labeled fluorescent components. In

the absence of NHERF1, the Fzd4-Vangl2 cross-correlation was negligible; in contrast, strong positive cross-correlation was observed after induction of NHERF1 expression (Fig. 23C). These results strongly support the hypothesis that NHERF1 scaffolds the formation of a complex between Fzd4 and Vangl2.

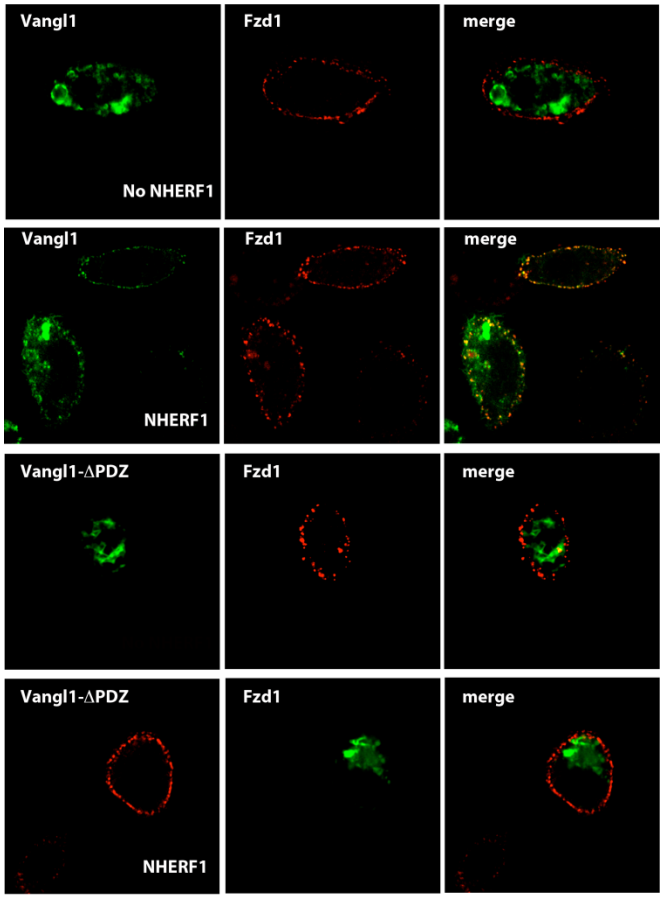

Figure 21. Vangl1 requires NHERF1 to traffic to the plasma membrane. CHO-N10 cells were co-transfected with HA-tagged Fzd1 and EGFO-Vangl1. NHERF1 expression was induced with tetracycline. After decorating the cells with Alexa594-anti-HA, the cells were imaged with a confocal microscope. **A)** Vangl1 co-localizes with Fzd1 at the plasma membrane only in cells that express NHERF1. **B)** A Vangl1 mutant in which the PDZ binding motif has been deleted does not traffic to the plasma membrane independently of the expression of NHERF

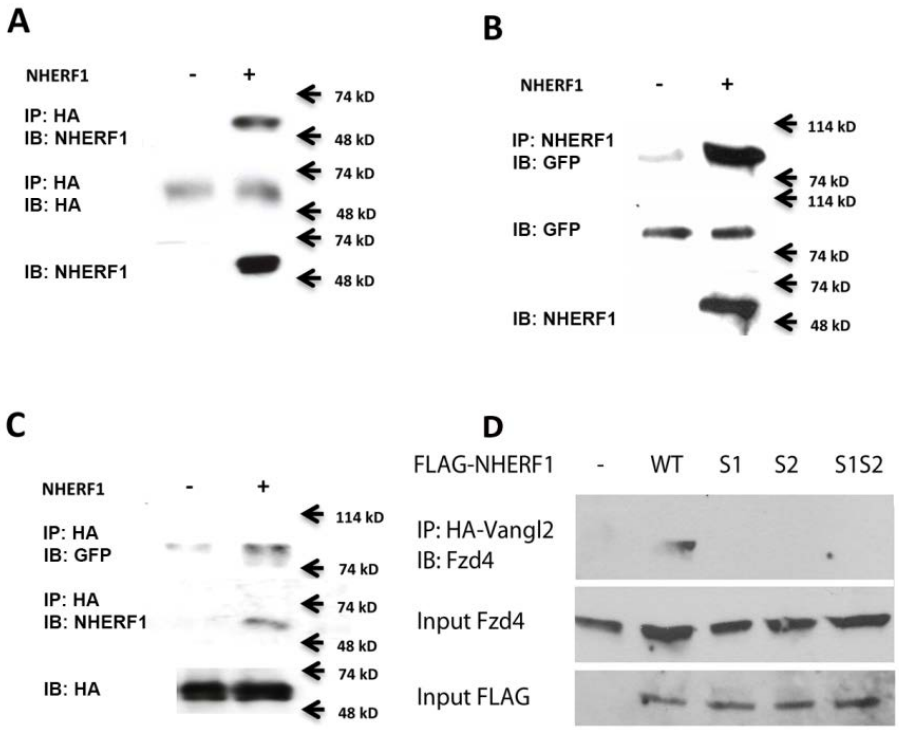

Figure 22. Fzd and Vangl2 bind NHERF1. **A)** CHO–N10 cells were transfected with HA-Fzd4, and the formation of a Fzd4-NHERF1 complex was determined by co-immunoprecipitation of the HA-tag followed by Western blotting with specific anti-NHERF1 antibodies. NHERF1 expression was induced by the addition of 100 ng/ml tetracycline. **B)** CHO-N10 cells were co-transfected with an EGFP-tagged Vangl2 construct and induced with either vehicle or 100 ng/ml tetracycline. After immunoprecipitation of NHERF1 using a specific antibody, the presence of the GFP label in the immunoprecipitated material was determined by Western blotting. **C)** CHO-N10 cells were co-transfected with HA-Fzd4 and EGFP-Vangl2. After induction with either vehicle or 15 ng/ml tetracycline, the HA-tagged material was immunoprecipitated, and the presence of the GFP tag in the co-immunoprecipitate was determined by Western blotting. **D)** Both PDZ domains of NHERF1 are required for the interaction of Fzd4 and Vangl2. Cells co-transfected with HA-Vangl2 and FLAG-tagged NHERF1 constructs (wt, S1, S2 and S1S2) were lysed and treated with anti-HA antibodies to immunoprecipitate Vangl2.

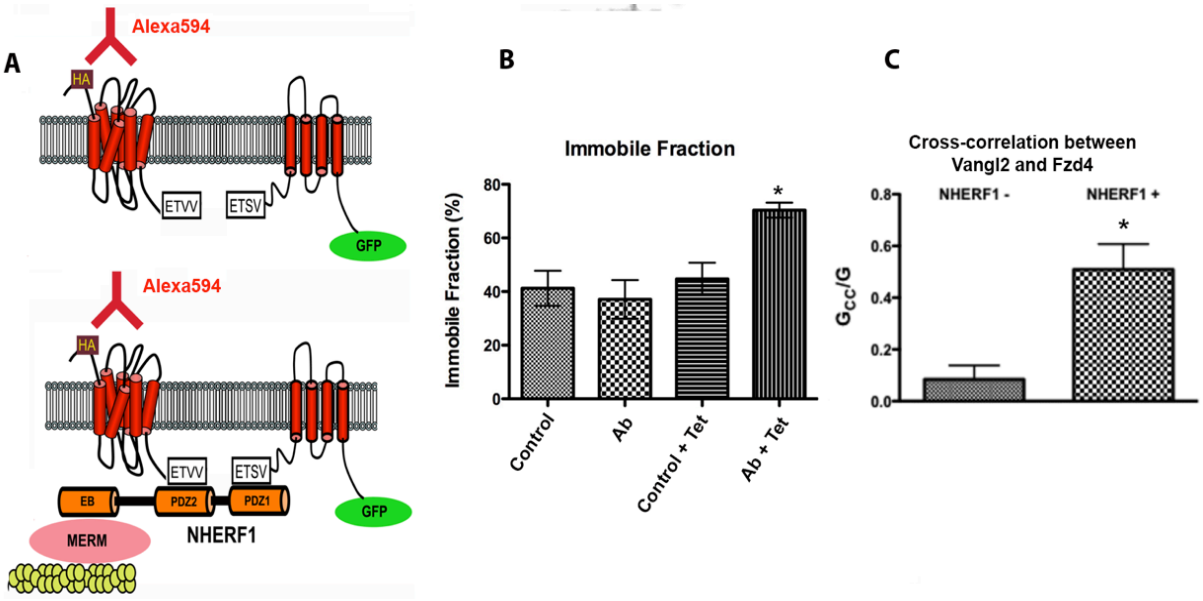

Figure 23. NHERF1 and Wnt receptors form a ternary complex: A) A model for the NHERF1-induced interaction of Fzd4 and Vangl2. We propose that Fzd4 and Vangl2 interact with the PDZ domains of NHERF1. In the absence of NHERF1, Fzd4 and Vangl2 interact only weakly; however, NHERF1 expression induces the formation of a Fzd4-Vangl2 complex. **B)** Addition of HA-specific and cross-linking antibodies immobilizes Vangl2 only when NHERF1 is expressed. CHO-N10 cells transfected with HA-tagged Fzd4 and EGFP-Vangl2 were induced with vehicle or 100 ng/ml tetracycline. Live cells were tagged with Alexa594-labeled anti-HA antibody (HA.11) followed by a secondary anti-mouse IgG cross-linking antibody. The mobility of EGFP-Vangl2 was determined by FRAP. **C)** Cross-correlation analysis of the formation of Fzd4-Vangl2 complexes. CHO-N10 cells co-transfected with HA-tagged Fzd4 and EGFP-tagged Vangl2 were incubated with Alexa594-anti-HA antibody. The autocorrelation function of EGFP-Vangl2 and the cross-correlation function of the EGFP label to the Alexa594 label were determined. The ratio of the cross-correlation (GCC) to the autocorrelation amplitudes is directly proportional to the fraction of Vangl2-Fzd4 complex formed.

3.4 DISCUSSION

PDZ proteins are ubiquitous molecular scaffolds that control multiple aspects of protein traffic and function. These regulatory roles are accomplished by the assembly and disassembly of multimeric complexes containing specific signaling and structural molecules that affect the distribution and functional characteristics of the target proteins. NHERF1 is one of the better-characterized members of this family. Initially identified as a regulator of the renal proximal

tubule brush border membrane Na^+/H^+ exchanger [22], NHERF1 serves a central physiological role in the regulation of mineral-ion homeostasis, including the regulation of parathyroid hormone receptor signaling [342-344]. A global NHERF1 knockout mouse was reported in 2002, and hydrocephalus was noted as a secondary phenotypic characteristic [39]. Our observations confirm the hydrocephalus phenotype of the NHERF1$^{-/-}$ mouse; moreover, we demonstrate that NHERF1 knockdown in zebrafish embryos also causes hydrocephalus, plus additional phenotypic issues not previously described in the mouse. Furthermore, we report here that the hydrocephalus caused by ablation of NHERF1 is associated with ciliary dysfunction in the ependymal lining of the cerebral ventricles.

How does NHERF1 modulate ciliogenesis? A direct, structural role for NHERF1 in the assembly of the cilium itself is unlikely. NHERF1 is excluded from the cilium [340] and the ependymal cells of NHERF1$^{-/-}$ animals contain cilia. Furthermore, the ependymal cilia of the NHERF1$^{-/-}$ mice have normal 9+2 structures, and are of normal length and diameter. A similar form of hydrocephalus linked to defective motile cilia was recently reported in mice missing the PCP genes Celsr2 and Celsr3. The main defect in the motile cilia of Celsr2$^{-/-}$ mice is deficient organization of the cilia, which results in impaired hydrodynamic CSF flow [198]. This defect was associated with anomalous planar polarization of the distribution of Vangl2, which failed to localize asymmetrically on the apical surface of the ependymal cells [198]. However, the defects caused by NHERF1 ablation are distinct from the typical PCP core gene knockouts, which involve primarily cilia disorganization without loss of cilia [345]. These differences may be linked to the increased β-catenin signaling resulting from NHERF1 ablation. A recent study linked increased levels of β-catenin to high expression of histone deacetylase (HDAC), leading to reduced tubulin acetylation and, therefore, impaired cilia formation [346]. Thus, NHERF1

appears to serve as a molecular router that regulates the relative input of Fzd-mediated signals to the canonical β-catenin pathway or to the alternative PCP pathway. In conclusion, we propose that the effects of NHERF1 are a consequence of two correlated activities: 1) inhibition of Wnt/β-catenin signaling; and 2) recruitment of Vangl proteins to the plasma membrane to promote PCP signaling. This latter role of NHERF1, viz regulating traffic of target proteins to the plasma membrane of polarized epithelia, is consistent with previous studies [347-350].

The direct regulation of Wnt signaling pathways by specific PDZ proteins has been documented. Many of the molecular components of the Wnt signaling pathways contain canonical PDZ domains or C-terminal PDZ-binding motifs, including 8 of the 10 mammalian Fzd receptors, β-catenin, and Vangl1/2 [91]. However, little is known about the role of these PDZ binding motifs and their interacting partners in the regulation of Wnt and PCP downstream signals. Because PDZ proteins are essentially scaffolds, the formation of multimeric complexes is a central feature of their role in the regulation of biological processes. Thus, the multi-PDZ protein MAGI-3 has been implicated in the stabilization of complexes containing Vangl2 and Fzd4/Fzd7 that localize to cell-cell contact sites [351]. We show here a similar scaffolding role for NHERF1 although with a very different subcellular distribution. NHERF1 is targeted to the apical end of polarized cells [91]. Inasmuch as our results demonstrate that Vangl2 is apical in wild type but not in NHERF1$^{-/-}$ ependyma, we conclude that NHERF1 is required for the apical localization of Vangl2.

The formation of Fzd-Vangl2 complexes has been postulated to play a central role in PCP signaling. Vangl2 interacts with Fzd3, but evidence for the interaction of Vangl2 with other members of the Fzd family is lacking [197]. We show here that NHERF1 promotes the interaction of Vangl2 with Fzd4 acting as a scaffold linking both proteins. This model provides a

mechanism for the regulation of PCP events by Fzd receptors that, unlike Fzd3, do not interact efficiently with Vangl2. Furthermore, this role of NHERF1 may not be unique. Recent reports suggest that other multi-PDZ proteins may also assemble protein complexes that include Fzd4 and Vangl2 [351]. Thus, several PDZ scaffolds may play redundant roles in coupling of PCP to diverse downstream functions depending on their relative levels of expression in specific cells and tissues. Our data demonstrate that NHERF1 is a critical PDZ scaffold involved in the coupling of the PCP pathway to the development of functional cilia.

We conclude that NHERF1 plays a crucial role in the regulation of β-catenin signaling, in the recruitment of Vangl proteins to the apical surface of polarized cells, in the formation of Vangl2-Fzd complexes, and in the proper development of cilia. To determine whether or not other ciliated organs were affected, we examined the upper respiratory pathways of wild type and knockout animals. We observed reduced numbers of cilia and ciliated cells in the trachea of knockout animals, although the defect was less dramatic than in the ependyma. Importantly, the ciliary defects observed in the tracheae of the NHERF1$^{-/-}$ animals were accompanied by a 30% decrease in fluid flow (Fig. 24). We conclude, therefore, that NHERF1 plays a general role in the proper orientation and function of motile cilia.

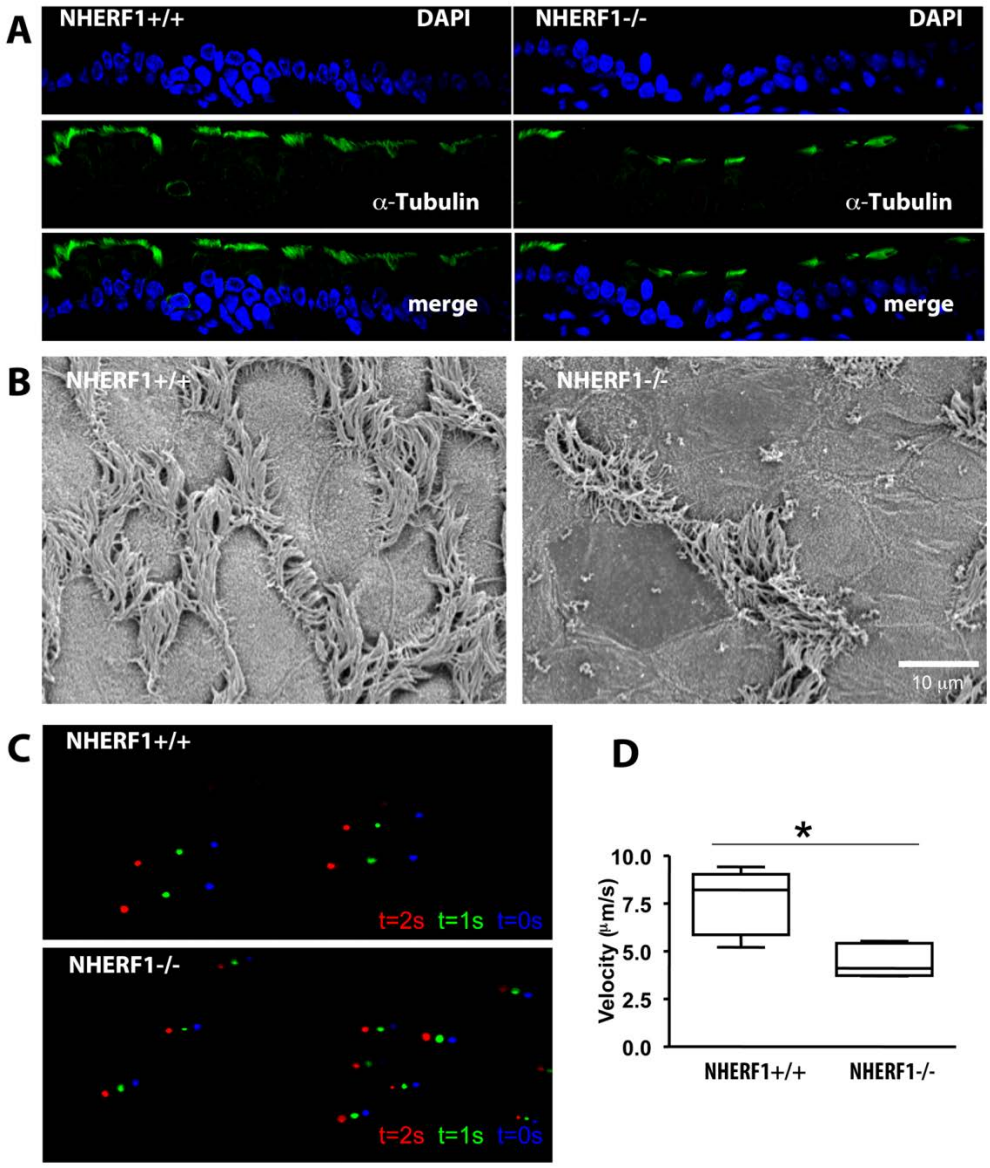

Figure 24. NHERF1 ablation alters cilia organization and function in the respiratory tract. A) Reduced number of motile cilia on the surface of the respiratory epithelium of NHERF1$^{-/-}$ mice. **B)** Scanning electron micrographs of the tracheae of NHERF1$^{-/-}$ mice shows patches of cells with no cilia. **C)** Ciliary function was determined in sagittal sections of the tracheae of NHERF1$^{+/+}$ and NHERF1$^{-/-}$ animals. Fresh sagittal sections were extended on a microscope slide. After addition of medium and 5μl of a suspension of fluorescent beads, a coverslip was placed on top of the sections and the movement of the beads was recorded by fluorescence microscopy using a 20X objective at 1-second intervals. The figure shows the relative positions of the beads using a color code (blue: time=0, green: time=1 second, red: time-=2 seconds). **D)** The average velocity of the microbeads was determined as described in *C*. * denotes statistical significance ($p<0.001$, N=3 independent experiments; at least 15 beads per experiment). Data for this figure was generated by Donna Stoltz and Guillermo Romero.

4.0 THESIS SUMMARY AND DISCUSSION

The focus of this thesis has been determining the role of the PDZ scaffold NHERF1 in the regulation of Wnt signaling in the ependyma and mammary tissues; more specifically Planar Cell Polarity and ciliogenesis in the ependyma and Wnt/β-catenin signaling in breast cancer.

Although several studies have implicated NHERF1 in the breast cancer progression, until now all of these studies have focused on NHERF1's regulation of PI3K/AKT signaling in the breast [307]. Recently, our laboratory reported that NHERF1 expression reduced β-catenin activity in breast cancer cells [40]. Based on the results of this earlier study, our group sought to determine the role of NHERF1 and Wnt signaling in mammary oncogenesis and progression. However, the results of the studies in chapter 2 were overall inconclusive. For one, MCF10As, which do not express NHERF1, could not be transformed through the expression of Wnt ligands, a result confirmed independently by a separate group [320]. It's possible the MCF10As have downregulated expression of the mammalian homologs of *porcupine* and *wntless*, resulting in improper Wnt ligand production and secretion. Unfortunately, we were not able to test if the Wnt transfected MCF10As were able to secrete the transfected ligands. MCF10As could also potentially have decreased Fzd or LRP5/6 expression.

Although we could not effectively test how NHERF1 expression effects Wnt-mediated transformation, we sought to determine if NHERF1 expression could contribute to endocrine resistance in breast cancer cells. As mentioned in previous sections, E2 expression stimulates

NHERF1 expression in breast cells and certain anti-estrogens such as tamoxifen and fulvestrant decrease NHERF1 expression in breast cancer cell lines. Therefore, we hypothesized that loss of NHERF1 through the inhibition of E2 signaling could lead to increased Wnt/β-catenin signaling and endocrine resistance. To test this hypothesis, we determined NHERF1 expression and sensitivity to the β-catenin inhibitor ICG-001 in endocrine sensitive (MCF-7 parental) and endocrine resistant (LTED, TamR, LY2) cell lines. Initial studies indicated that the proliferation of endocrine resistant cell lines was significantly inhibited by ICG-001, while endocrine sensitive cells were not affected.

Our results indicate that acute deprivation of E2 in parental MCF-7s significantly reduced NHERF1 expression, consistent with previous reports from independent groups [162]. Unexpectedly, the endocrine resistant cell lines expressed NHERF1 at levels comparable to those of the parental cell lines cultured in the presence of excess E2. Human IHC results indicated that at least in some cases chronic anti-estrogen treatment can decrease NHERF1 expression, yet in other cases NHERF1 expression increases or stays the same. One explanation for this discrepancy between acute and chronic estrogen deprivation is that over time cells may increase NHERF1 expression through non-E2 mediated signaling pathways. In colon cells, mTOR inhibition by rapamycin significantly decreased NHERF1 expression [326]. The mechanistic target of rapamycin (mTOR) is an atypical S/T kinase that functions downstream of many different signaling pathways, including growth factors, hormones and cytokines [352]. NFκB signaling has also been shown to increase NHERF1 expression [325]. As mentioned in chapter 1, ERα can inhibit NFκB signaling in the nucleus. Therefore, it is possible that the decrease of NHERF1 expression caused by loss of ERα signaling is compensated by increases in NFκB activation. Others have reported increased NFκB signaling in estrogen receptor negative breast

cancer cells, further supporting this hypothesis [220]. However, more evidence is needed to determine the factors that increase NHERF1 expression in estrogen-independent MCF-7 derived cell lines.

Later experiments revealed that ICG-001 inhibits the growth of some endocrine sensitive breast cancer cell lines, parental T47Ds and MCF-7 ATCC cells, despite the presence of estrogen. Additionally, no significant differences in β-catenin phosphorylation status and expression could be found between endocrine sensitive and endocrine resistant MCF-7 cells. It is possible that in breast cancer cells in culture β-catenin transcription contributes to cell proliferation in the presence of estrogen, but it is not always needed, explaining the lack of correlation between endocrine resistance and ICG-001 sensitivity. Another confounding factor is the fact that one of the main differences between the MCF-7 and MCF-7 ATCC cell lines used was the initial passage number (155 vs 51 respectively), which could have contributed to the differences seen in our studies. Different culturing conditions between laboratories, cell line passage number, and a lack of authentication of cell lines often leads to conflicting results for the same assay [353]. For example, MCF-7s cultured in different laboratories have been reported to have a wide range of sensitivity to estrogen and different growth rates [354, 355]. Indeed, misidentification of cell lines and other factors that contribute to the differences between theoretically identical cell lines have been recognized as a major issue in scientific research [356].

The results from our study demonstrated that Wnt conditioned media inhibits raloxifene but not toremifene induced growth arrest. Acute toremifene treatment did not significantly reduce NHERF1 expression, yet high concentrations of raloxifene reduced NHERF1 by over 60%. However, one of the characteristic features of SERMs is their ability to assert different

signaling effects depending on the target tissues and context of ERα co-regulators [357]. Recent proteomic studies revealed that tamoxifen, raloxifene and fulvestrant have significant differences in their regulation of ERα-cofactor interactions [358], however more evidence is needed to determine the mechanism behind the observed differences between raloxifene and toremifene Wnt rescue.

Although the role of NHERF1 and Wnt/β-catenin signaling in breast cancer progression remains unclear, several strides have been made in understanding the role of NHERF1 in Wnt/PCP signaling in the ependyma. Our studies confirmed the presence of a hydrocephalus phenotype in NHERF1 knock out mice that was originally reported in 2002. We determined that NHERF1 knockout mice developed a form of communicating hydrocephalus characterized by reduced ciliogenesis and random organization of cilial bodies in the ependymal tissues. Furthermore, we showed that NHERF1 knockdown in zebrafish also leads to hydrocephalus, demonstrating that NHERF1 expression can regulate motile cilia function in more than one species. Since NHERF1 is excluded from the cilia structure, we hypothesized that the phenotypes associated with loss of NHERF1could be due to deregulation of the Wnt PCP pathway. As reviewed in chapter 1, Wnt PCP signaling has been linked to ciliogeneisis and cilia organization by several independent groups, and mice lacking expression of the PCP proteins Celsr2 and Celsr3 show a similar hydrocephalus phenotype. This hypothesis was further supported by the observation that NHERF1 knockdown in zebrafish presented tail deformities similar to those of the Wnt5a/*pipetail* phenotype in zebrafish and reminiscent of the characteristic phenotype of *Vangl* Looptail mutants in mice.

Further evidence that NHERF1 could potentially regulate Wnt PCP signaling came from the observation that NHERF1 knockout mice lacked Vangl2 expression at the apical surface of

the ependyma. Vangl1 & Vangl2 both possess class I PDZ ligands at their C-termini that can mediate interactions the PDZ domains of NHERF1. Our results indicate that NHERF1 interacts with Vangl receptors and promotes their plasma membrane localization. Furthermore, mutation of Vangl C-terminal PDZ ligands results in loss of Vangl plasma membrane subcellular localization. Vangl proteins can also interact with other PDZ scaffolds, including PSD-95 and MAGI-3 [351, 359]. Studies have shown that accumulation of Vangl2 at the plasma membrane during certain stages of gastrulation is crucial for initiating convergent extension in zebrafish, and that Vangl2 subcellular localization is dynamic and highly regulated [360]. These results are consistent with several studies that implicate PDZs in the maintenance of apical-basal polarity. PDZ proteins themselves are polarized in their distribution, and specific PDZ domain containing proteins are rarely found on both apical and basolateral membranes [361]. PDZs are also required for the plasma membrane targeting of their targets. PDZs are involved in the trafficking and sorting of their binding partners, as well as retaining their targets in their final destination [33, 361, 362]. If PDZs themselves are polarized, than it stands to reason that a primary function of PDZs is to properly localize their targets to either apical or basolateral membranes, thereby aiding in the maintenance of apical-basal polarity [361].

PDZs also have more active functions in the determination of polarity. Scribble is a PDZ domain containing scaffold that has also been reported to interact with PCP proteins and regulate their signaling [363, 364]. Scribble is located at septate junctions on the lateral plasma membrane of epithelial cells, right at the apical-basal border. Scribble regulates apical-basal polarity in *Drosophila* and loss of the protein leads to severe defects [365]. Vangl and Scribble have been shown to interact genetically in *Drosophila* and bind directly *in vitro.* Although Scribble is not required for the apical localization of Stbm (Vangl homolog), mutation of

Scribble at the Scribble-Stbm interaction domain results in PCP defects [366]. The authors of this study hypothesize that Scribble could act as a downstream PCP effector, but it is also possible that Scribble is required for the asymmetric distribution of other components of PCP signaling. Indeed, planar polarity cannot be achieved if core PCP components are not asymmetrically expressed and they rely on each other for proper localization [367].

Aside from their function in trafficking and localization, PDZ proteins act as critical scaffolds in cell signaling [368]. Studies in cell culture models indicated that NHERF1 expression induces the association of Vangl2 with Fzd receptors, and that this function requires both PDZ domains of the scaffold. The PDZ domain containing scaffold MAGI-3 has also been shown to induce the formation of Fzd-Vangl complexes, suggesting that PDZ scaffolds could be crucial for the regulation of Wnt PCP signaling complexes [351]. Given that NHERF1 knockout mice also presented increased nuclear β-catenin in the ependyma, these results portray NHERF1 as a master regulator of Wnt signaling in the mouse ependyma. We propose that NHERF1 may function as a molecular switch between the two pathways, inducing complexes that favor Wnt PCP signaling at the expense of β-catenin activation. This model is further supported by the observation that NHERF1 knockout in mice reduces cilia number, a phenotype not associated with Vangl knockouts. This difference can be explained by the fact that increased levels of β-catenin reduce tubulin acetylation and impair cilia formation, further supporting the model that NHERF1 regulates both canonical and non-canonical signaling [346].

Many of the Wnt signaling components contain C-terminal PDZ ligands: 8 of the 10 Fzd receptors (all except Fzd3 and Fzd6), Vangl1/2 and β-catenin. Furthermore, Dvl contains a PDZ domain which mediates its' interaction with Fzd receptors. Dvl mediates both Wnt/β-catenin and Wnt PCP signaling, raising the question of how Dvl "decides" which pathway to activate.

So far the evidence suggests that the initial events that determine which pathway is activated are mediated by the formation of protein complexes at the plasma membrane. Wnt/β-catenin signaling is initiated by the formation of protein complexes termed signalosomes that contain Fzd and LRP5/6 receptors [369]. Preceding PCP signaling events in *Drosophila*, two complexes are located on opposite sides of the plasma membrane; Fz-Dsh-Dgo complex localized distally and Stbm-Pk localized proximally, the interaction between distal and proximal PCP components in adjacent cells then propagates the PCP signal along the tissue [369]. In zebrafish, the protein Rack1 is required for plasma membrane localization of Vangl2, and its expression antagonizes Wnt/β-catenin signaling [370]. In this way, factors that favor the formation of Wnt PCP complexes seem to simultaneously decrease the activation of Wnt canonical signaling. This suggests that certain protein complexes are formed at the expense of others, consistent with our data.

We conclude that NHERF1 aids in the plasma membrane targeting of Vangl proteins in certain epithelial tissues, and that it further acts as a scaffold for Wnt PCP signaling complexes. Binding of NHERF1 to Fzd receptors increases Fzd-Vangl association and results in reduced β-catenin activation. The lack of a global phenotype indicative of Wnt dysfunction in NHERF1 knock out mice suggests that the function of the scaffold is redundant in certain contexts. This implies a general function for PDZ proteins in the regulation of Wnt-signaling complexes and establishment of polarity.

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